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A study on the stability of urinary free catecholamines and free methyl-derivatives at different pH, temperature and time of storage

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Abstract

Bakground: The goal of our study was to test the relative stability of urine, unconjugated, free catecholamines and the methyl derivatives. We measured the change in concentrations in commercially available urines after storage at various pH values, temperatures and time, from days up to 10 weeks.
Methods: Samples of commercial control urines were adjusted to pH 2.0, 4.0, 6.0 and 8.0 and aliquots stored at ambient temperature (20–26°C), 4°C and −18°C. The free catecholamines (cats) and the free methyl derivatives (mets) were measured after 1, 2, 3 and 6 days and 1, 2, 3 and 10 weeks using the automated sample trace enrichment dialysis (ASTED) procedure with reversed phase ion pair high performance liquid chromatography (HPLC) and coulometric detection.
Results: Free catecholamines were relatively stable, with $<15\%$ loss of concentration, when stored at pH 6.0 or less for at least 4 days and up to 10 weeks at pH 2.0 at either 4°C or −18°C. At pH 8.0, the concentration fell to $<60\%$ after 48 h and at a pH of 6.0 or 8.0, up to 90% was lost within the first week at 4°C and 25°C. More than 40% of free normetadrenaline and metadrenaline were lost after 1–2 weeks when stored at 20–25°C and pH 8.0. After 10 weeks at pH 4.0, 6.0 and 8.0, up to 90% loss was observed at 25°C. Free cats were stable at pH 2.0 and 4.0 at −18°C and the free mets were stable at −18°C over the entire time period studied and at all pHs.
Conclusions: In the analysis of free catecholamine and the free methyl derivatives, urine samples should be acidified to a pH range 2.0–3.0 to ensure stability and hence the correct analysis.
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Introduction

The measurement of the free (non-conjugated) or total (combination of the free and conjugated) individual catecholamines (cats) and/or the total individual methylated derivatives (mets) is well established for the detection of tumours that secrete catecholamines in urine (1–3) or in plasma (4). Recently, measurement with on line clean up [automated sample trace enrichment dialysis (ASTED)] and ion pair based separation of the free methylated compounds was suggested to be at least as good as total individual mets and/or free unconjugated parent (cats), or possibly a better alternative (5). Over the past several years, we have successfully used the ASTED procedure for the measurement of both the free parent and methylated derivatives. We have always observed good concordance between the two; if one is increased the other is also increased (6). However, cases that break the rules can always be found. For example, normal cats raised mets and normal cats and mets with increased vanillylmandelic acid (VMA) (7). As many analytes as possible are used to catch all the possible scenarios, although for the diagnosis of phaechromocytoma, urinary free metadrenalines have been shown to demonstrate superior clinical sensitivity over plasma or urinary catecholamines or urinary VMA (8). Also, we have confirmed the importance and benefit of simultaneous measurement of dopamine, which can be increased in $\sim 3\%$ of urines from adults with catecholamine secreting tumours (9, 10). A recent review on tumours that secrete catecholamines suggested that metadrenalines in urine or plasma are the most likely to be abnormal (11). However, this is clearly not correct in patients with tumours that secrete dopamine only, or its metabolites (9, 10).

The underlying problem for all these analyses is the integrity/dependability of the sample. Catecholamines are sensitive to oxidation, particularly at greater than neutral pHs where the well described adenochrome-like compounds are formed from adrenaline and noradrenaline, and dopachrome from dopamine (12). Thus, it is imperative that low pHs of <4.0, and preferably <3.0 are used, along with an antioxidant for long-term storage. A major confounding variable in the diagnostic value of urinary free cats is changes due to oxidative decay; a loss of as little as 20%–30% can result in abnormal values being reported as normal. This is another

Table 1 Analytical characteristics of free catecholamines and free methyl derivatives in commercial quality control urine.

Analyte, nmol/L	Noradrenaline	Adrenaline	Normetadrenaline	Metadrenaline	Dopamine
Lypho 1	250	74	1385	362	552
	(210–290)	(54–94)	(1125–1645)	(262–462)	(452–652)
	7.0%	9.6%	8.3%	11%	7.4%
Lypho 2	1158	466	7878	2850	3124
	(1028–1278)	(386–546)	(6478–9278)	(2210–3490)	(2624–3624)
	4.8%	6.7%	7.3%	9%	5.7%
BioRad HPLC					
Level 1	284	93	1911	598	601
	(213–355)	(60–126)	(1420–2402)	(446–750)	(451–751)
Level 2	1229	502	8518	3093	3461
	(969–1489)	(393–612)	(6825–10,210)	(2459–3726)	(2612–4310)

Lypho 1 and 2 values are nmol/L as mean and in parentheses ( ) ranges observed over a 6-month period (n = 20); the CV% quoted is between batch precision analysis. The BioRad (Lypho) HPLC values are those given by the manufacturer showing the target mean and range of acceptable results in ( ) and indicate similar values to those analysed. The slightly lower values for the Lypho 1 normetadrenaline and metadrenaline probaly related to the amount of endogenous sulpho complex not measured.

reason why measurement of mets is preferred, as they are apparently much less likely to degrade at neutral pH. A recent study (13) of stability over 7 days indicated that the parent catecholamines are relatively unstable in unacidified urines, losing more than 50% of their initial concentration. However, total (free plus conjugated) methyl compounds remained stable over the 7 days. These findings have been confirmed several times, in particular for the non-methylated and unconjugated parent compounds (14, 15). However, from our own experience in the measurement of the unconjugated free compounds, it is often observed that low cats are present with low mets in unacidified urine with pHs up to pH 8.0. This finding suggests that free mets are not as stable as predicted.

If the assay of the free compounds, particularly free mets becomes more widespread, it is important to have good analytical data on their relative stabilities in urine. Our goal was to reassess the stability in urine of both unconjugated free and methylated compounds, over a pH range of 2.0, 4.0, 6.0 and 8.0, temperatures of –18ºC, 4ºC and room temperature (20–26ºC), and over a long period of time (days up to 10 weeks). We chose a reference urine material so that we could be certain of the concentrations (added in as the free metabolites) and little variation in antioxidants such as vitamin C that could affect stability.

Materials and methods

All chemicals used were of Analar grade and solutions were prepared using doubly deionised water (Ultra Q, Elga Products, High Wycombe, UK).

Urine Lypho I and II (Lyphocheck, Urine Quality Controls, BioRad Laboratories, Hemel Hempstead, UK) were used for comparative purposes, with the stated and laboratory measured concentrations for the relevant analytes shown in Table 1. The commercial urine samples were spiked with unconjugated mets. The values quoted in Table 1 for the BioRad high performance liquid chromatography (HPLC) are total methylcated derivatives and include any residual urine derivative compounds; hence the slight discrepancy between the stated values and our measured concentrations. Aliquots of these urines were prepared at various pH values 2.0, 4.0, 6.0 and 8.0 by the addition of small amounts of 4 M sulphuric acid (H_2SO_4), or 4 M sodium hydroxide (NaOH). The amounts added did not cause any significant dilutional effects. However, to avoid any misinterpretation due to dilution, data were expressed as percent of the analysis obtained at day 0. The samples were stored at ambient room temperature (20–26ºC), 4ºC and –18ºC and analysed on days 0, 2, 4, and 6 and weeks 1, 2, 3 and 10.

Separate aliquots were measured for the free catecholamines, noradrenaline, adrenaline and dopamine, and the free methyl derivatives (mets) normetadrenaline and metadrenaline. The analysis (16) of all these separate compounds was achieved using ion pair HPLC on a 15 cm Spherisorb 5 octadodecyl silanyl (ODS) (2) after automated sample preparation (ASTED) and detection by coulometry (Coulochem, ESA Analytical Ltd., Aylesbury, UK).

The actual analytical between batch imprecision for each analyte in Table 1 was between 4.8% and 11%, with an average of ~7.5%. A reduction in concentration was regarded as significant if the measured change was >15%, determined by taking two times the average CV of 7.5% as a cut-off threshold for significant change. The data are presented graphically with individual data points to show changes at the different times and temperatures.

Results

The free catecholamines were relatively stable at pH 6.0 or less for at least 4 days, with less than a 15% decrease in concentration as shown in Figures 1–3 (urine Lypho 1 and 2). However, at pH 8.0, the measured concentration fell to <60% of starting values after 48 h. Even when stored at –18ºC at high pH, the change in concentration was still significant with more than 60% loss after 4 days. Studies with Lypho 2 urine that contained higher amounts, the free catecholamines degraded in similar fashion after 2–3 days at ambient temperature and pH 8.0. Following 1 week of storage at either pH 6.0 and 8.0, all the free analyte was lost. There was no significant change in the measured concentration of free normetadrenaline and metadrenaline over a 7-day period at any pH when stored at 4°C or –18ºC. However,

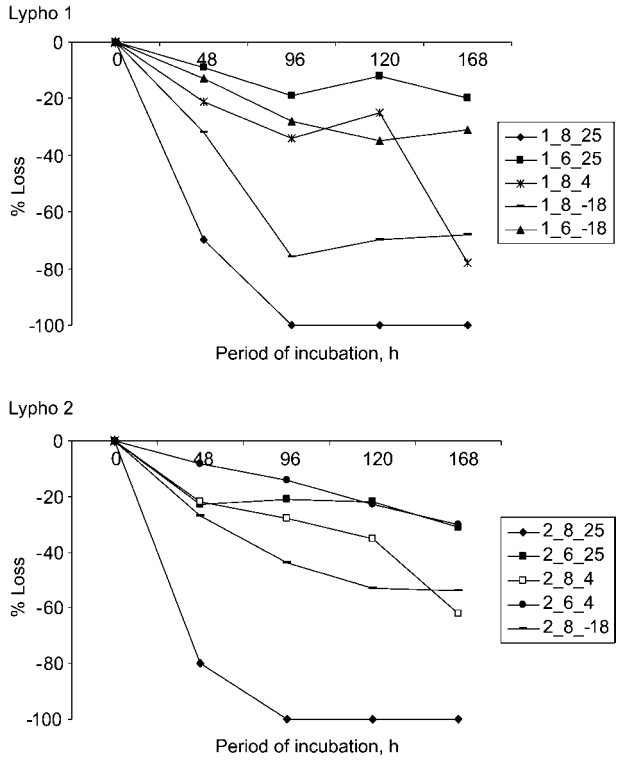

Figure 1 Change in concentration of noradrenaline in urine Lypho 1 and Lypho 2 after storage up to 7 days at various temperatures (25°C, 4°C and −18ºC).
Samples kept at pH 2.0 or 4.0 and temperatures of 4°C or −18ºC showed no significant change in the measured concentration.

when stored at pH 8.0 and ambient/room temperature (25ºC), normetadrenaline showed a decrease in concentration of up to 20% after 7 days, while metadrenaline showed no change (data not shown).

Studies of longer storage times (Figure 4) showed that free catecholamines were stable at pH 2.0 and pH 4.0 for up to 10 weeks at either 4°C or −18ºC. However, at pH 6.0 and 8.0, 90% was lost within the first week at 4°C or 25ºC. Marked loss was also observed at pH 4.0 when stored at 25ºC for up to 10 weeks.

Free normetadrenaline (Figure 5) showed more than 90% loss at pH 8.0 when stored at 25ºC for 2 weeks, and from 40% to >90% loss at 20ºC after 10 weeks at pH 4.0, 6.0 or 8.0. Free metadrenaline (Figure 6) showed more than 75% loss after 10 weeks at pH 4.0, 6.0 or 8.0 when stored at 25ºC.

Fee normetadrenaline and metadrenaline in both Lypho 1 and 2 were stable when stored at −18ºC over the entire time period and at all pH values.

Discussion

This study showed that free catecholamines are relatively stable over several days and a range of concentrations if the urine is kept at 4ºC and at pH 6.0 or less. This has also been observed (17) with urine collected in the hospital ward without acid preservative and pH values up to 7.0, as long as samples were delivered to the laboratory and analysed immediately or acidified and stored at −18ºC prior to analysis. To ensure stability of free parent and methylated catecholamines, we always recommend collecting urine in bottles containing acid. Following receipt of the sample in the laboratory, the urine pH is checked and acidified if necessary to at least a pH of 3.0 and stored at −18ºC before analysis within 1–2 weeks. The dependability of sample integrity is probably one of the confounding variables in the diagnostic value of free catecholamines, and is one of the major reasons why measurement of the mets is preferred; they are much less likely to degrade at neutral pH. However, we have now shown that the free mets are also prone to degradation if maintained at high pH and room temperature. Thus, precaution must be taken to avoid loss of these analytes from oxidation. It may of course be that the sulphated forms of the mets are themselves more stable with long-term storage regardless of pH, although no studies on this aspect have been reported.

Studies with individual human samples, as opposed to commercially available control urine, could be confounded by the presence of antioxidants such as vitamin C, citric acid and other amino acids that might act as oxidative scavengers. This probably explains why the change in urine cats can be minimal for up to 2 days, even at room temperature (13). This latter study showed that catecholamines were stable at pH 7.0 and 4ºC, whereas after 7 days at room temperature

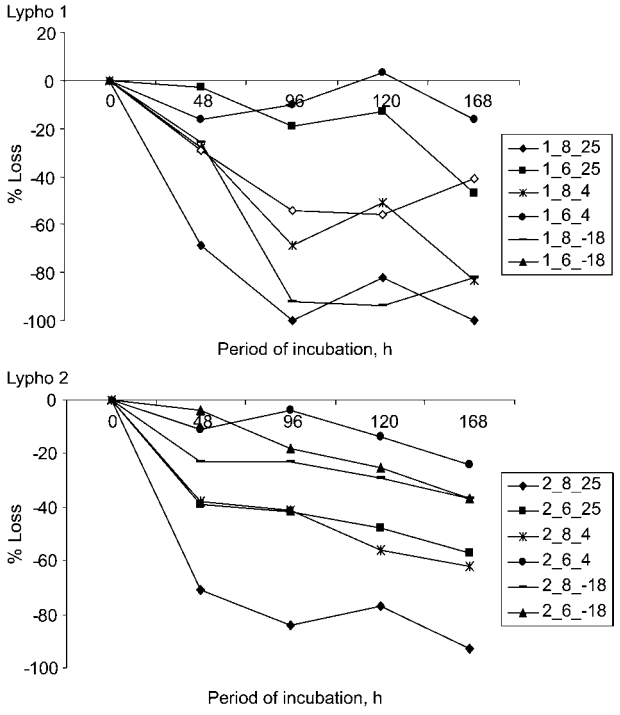

Figure 2 Change in concentration of adrenaline in urine Lypho 1 and Lypho 2 after storage for up to 7 days at various temperatures 25°C, 4°C and −18ºC.
Samples kept at pH 2.0 or 4.0 and temperatures of 4°C or −18ºC showed no significant change in the measured concentration.

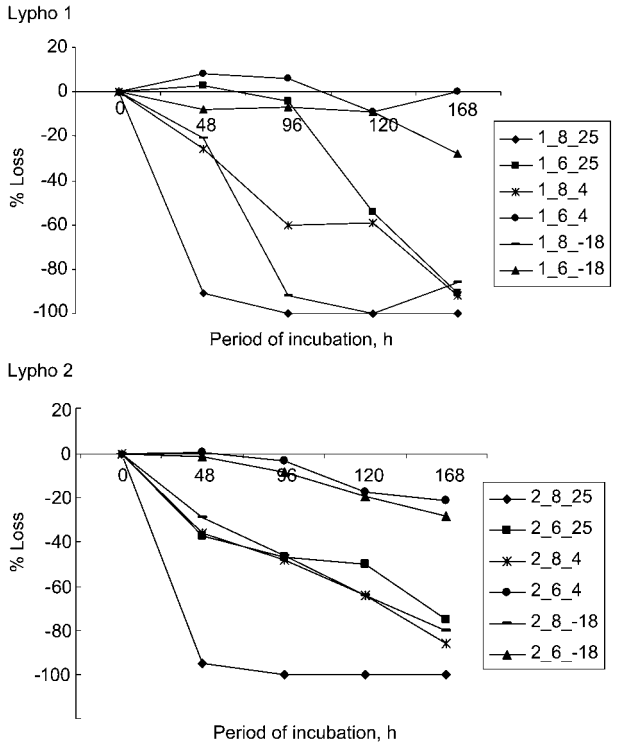

Figure 3 Change in concentration of dopamine in urine Lypho 1 and Lypho 2 after storage for up to 7 days at various temperatures 25°C, 4°C and −18°C.
Samples kept at pH 2.0 or 4.0 and temperatures of 4°C or −18°C showed no significant change in the measured concentration.

(20–25°C) a loss in concentrations of up to 30% was reported. The free catecholamines were relatively stable for a few days at pH 8.0, but only if kept at 4°C (13). At the extreme pH of 10.0, catecholamines degraded within a few hours. Interestingly, at a pH of <1.0, measured free catecholamines increased up to 50% after 7 days due to hydrolysis of the conjugated forms (13). Also, over-acidification can cause urine to become extremely dark in appearance, resulting in analytical problems due to extraneous peaks on the chromatogram. Therefore, it is important not to acidify to a pH of <2.0.

A recent review (18) on the analysis of urinary catecholamines concluded that any strong acid could be used as long as the final pH was 3.5 or less. However, for assays using the ASTED procedure, H_2SO_4 is recommended as preservative because this acid, unlike hydrochloric acid is non-oxidising (16). To reduce the risk to patients posed by the presence of acid in urine collection bottles, the addition of sand (1 g Sigma grade for a 24-h collection) has been used to maintain a low urine pH without risks of acid leakage (18). An alternative to the use of a strong acid preservative was the use of formate buffer (19) to maintain the urine pH at $\sim$3.5. Urine catecholamines preserved in this way were as stable as those in acid preserved urines when stored at −80°C. However, decrease in concentration of up to 40% was observed after storage for 8 weeks at 4°C. Alternative stabilising agents such as EDTA or sodium bisulphite could be used without any loss of activity, as long as samples were collected and then stored immediately at −18°C before analyis (13). The effect of physiological temperature should also not be ignored as urine may reside in the bladder at elevated pH and 37°C for several hours. Under these conditions, instability may be increased particularly for the catecholamines (20). These authors therefore recommended that during urine collection for measurement of catecholamines, the bladder should be emptied every 3 h, if possible.

Because of the problems associated with the collection of urine into acid, there is increasing support for measurement of methylated catecholamine derivatives as the most appropriate – 'best test' – for catecholamine secreting tumours. However, we advise that both types of compounds be measured, especially considering that the diagnostic features of these tumours are the excess production of active biogenic amines, and not the methyl derivatives (21). However, a recent review on the function and metabolism indicated that the free methylated compounds may show some physiological function (22), whereas sulpho conjugation (the major urine metabolite) results in complete deactivation of the catechols (23). Of interest is that a significant proportion of methylation can occur within the adrenal gland and the tumours themselves, and it is possible to relate the amount of methyl derivatives that are secreted to tumour size (8, 24). These arguments support the view that the free species are the most physiologically relevant and therefore the most appropriate analytical target.

The value of measurements in plasma (3) of the free non- and methylated components really depends on improvements in analytical performance, appropriateness of sample collection and good renal function. If these conditions can be satisfied, such assays will have marked impact in terms of convenience to both the patient and laboratory. However, at this time most laboratories still use urine collection as the basis in the investigation of tumours that secrete catecholamines. Unfortunately, whatever test is used, it is still possible that both misleading laboratory results combined with clinical investigation including CT and magnetic resonance imaging (MRI) may miss the tumour or result in the wrong diagnosis, as shown in a recent review on the diagnosis of phaechromocytoma (25). The patient's age may also be a confounding variable (26). In spite of these issues, a recent study (27) suggested that plasma free metanephrines are most likely to confirm the presence of a catecholamine secreting tumour (being up to 100$\times$normal concentrations), rather than urine mets, cats or HMMA (4-hydroxy-3-methoxy mandelic acid). Unfortunately, the concentrations of the separate mets are still relatively low, requiring up to 1 mL of plasma for analysis by HPLC, and also can be raised up to 10 times normal in patients with renal failure. In addition, the analytical performance at low concentrations can be poor, as seen in our own experience of an automated plasma catecholamine method that used electrochemical detection where the analytical performance was unpredictable (28). However, newly developed liquid chromatography-mass spectrometry

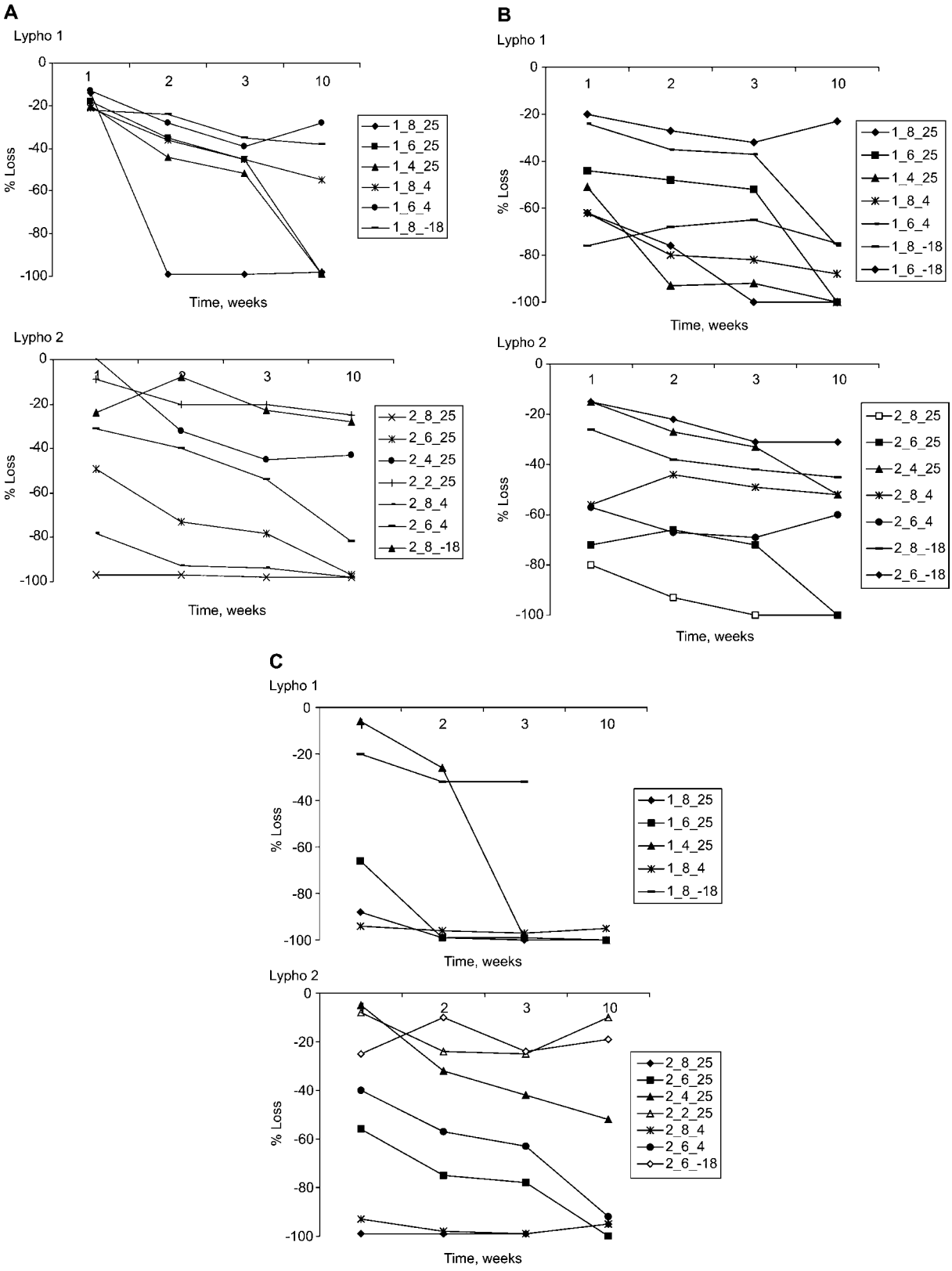

Figure 4 Change in concentration of (A) noradrenaline, (B) adrenaline and (C) dopamine in urine Lypho 1 and Lypho 2 after storage from 1 to 10 weeks at various pH values and temperatures of 25°C, 4°C or −18°C.
Samples kept at pH 2.0 or 4.0 and temperatures of 4°C and −18°C showed no significant change in the measured concentration.

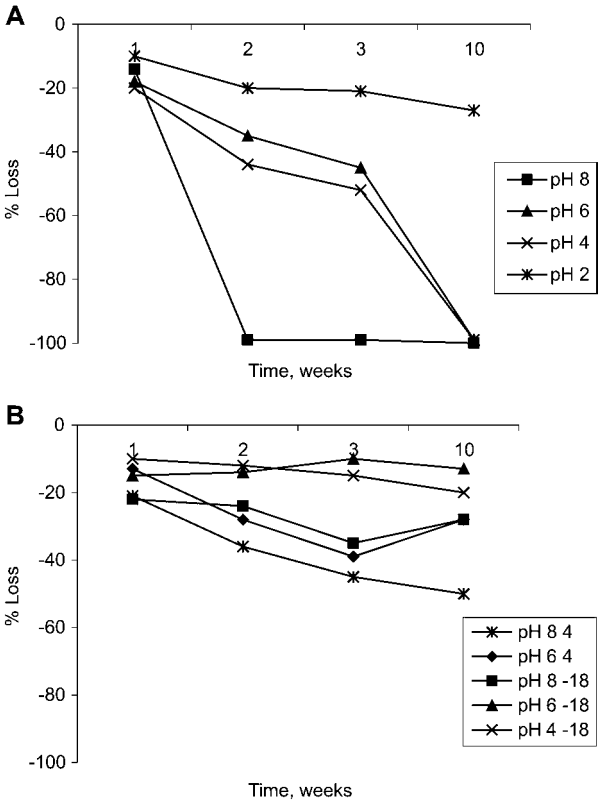

Figure 5 Change in concentration of (A) normetadrenaline at 25ºC and (B) normetadrenaline at 4°C in Lypho 1 after storage from 1 to 10 weeks.
Lypho 2 showed very similar trends, but data are not shown. Samples kept at pH 2.0 and or 4.0 and temperatures of 4°C and –18ºC showed no significant change in the measured concentration.

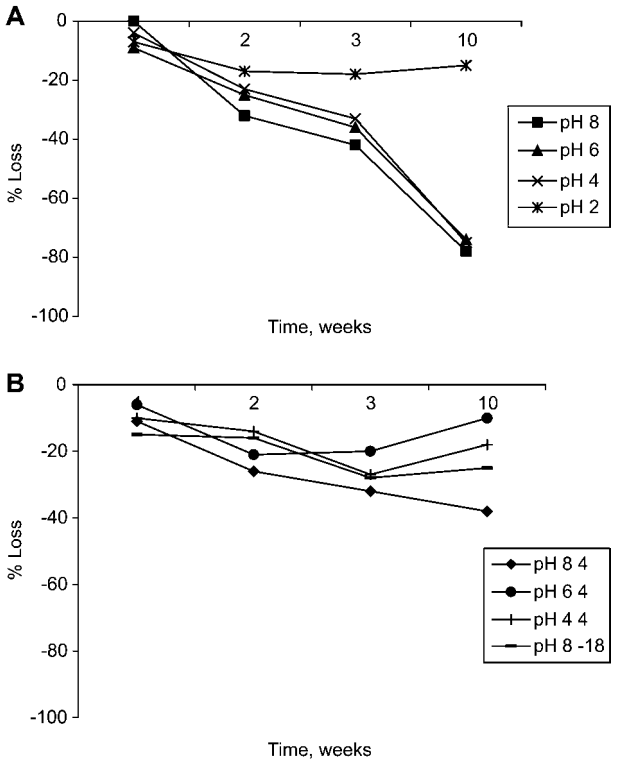

Figure 6 Change in concentration of (A) metadrenaline at 25ºC and (B) metadrenaline at 4°C and –18ºC in Lypho 1 after storage from 1 to 10 weeks.
Lypho 2 showed very similar trends, but data are not shown. Samples kept at pH 2.0 or 4.0 and temperatures of 4°C and –18ºC showed no significant change in the measured concentration.

(LCMS) techniques require less sample (50–100 μL) and seem to provide good analytical performance both at normal and increased values (29). Nevertheless, increased plasma metanephrines may be misleading in up to 20% of patients (25). Indeed, a recent review concluded that plasma and urine free metanephrines should be considered complementary rather than mutually exclusive (30). Thus, it is important that the analytical pitfalls, in particular the instability of the analytes and the need for appropriate collection, are clearly understood.

In conclusion, we confirmed that free catecholamines can be stable in urine at a pH of 6.0 (i.e., similar to an unacidified urine collection) for short periods of time such as 2–3 days, and particularly if stored at 4ºC or –18ºC. The free methylated derivatives are stable for up to 2–3 weeks if stored under similar conditions, but do degrade if urine is kept at a high pH of up to 8.0 for several days/weeks, particularly if left at room temperature. Therefore, to avoid any problems with analyte instability, it is strongly recommended that 24 h urines be collected into acid, or to acidify freshly collected samples to between pH 2.0 and 3.0 and then store at –18ºC prior to analysis. Care must also be taken not to overacidify samples to a pH of <2.0 to avoid hydrolysis of the conjugated compounds. Our view is in agreement with that of others (5, 8) that analysis of both the free cats, and including dopamine and free mets will provide the most diagnostically useful profile.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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