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Determination of allantoin in biofluids using micellar electrokinetic capillary chromatography

Luwiza N. Alfazema[a,*], Sian Howells[a], David Perrett[b]

[a]*Chemical Life Sciences Dept, University of Greenwich, Wellington Street, London SE18 6PF, UK*
[b]*Department of Medicine, St Bartholomew's Hospital, London EC1A 7BE, UK*

Abstract

A micellar electrokinetic chromatographic method is described for the determination and quantitation of allantoin, an end-product of purine metabolism in mammals that is applicable to biofluids of different mammal species and man. The method was optimised following a study on the effect of pH and sample preparation procedure. Final conditions were 30 m*M* sodium tetraborate, pH 9.5, 75 m*M* sodium dodecyl sulphate, 20 kV and 20°C. Allantoin was well resolved from endogenous compounds and could be determined in horse, dog, mouse and rabbit urine. No allantoin could be found in man. No complicated sample treatment was necessary, thus the developed method was rapid (<5 min), sensitive (5 μ*M*) and simple. Results from this work will permit the determination of allantoin in man as a measure of free radical generation reactions as well as its presence in the plasma and other biofluids with modification of the sample preparation procedures.

Keywords: Allantoin; Uric acid

1. Introduction

Allantoin is a natural end-product of purine degradation in most mammals. In man and primates, due to lack of the enzyme uricase (E.C.1.7.3.3), the end-product of this catabolic pathway is uric acid (Fig. 1).

Allantoin is difficult to measure due to lack of the characteristic UV spectra of purines at around 260 nm. Determination of allantoin in biofluids has been based on the Rimini–Schryver reaction; allantoin is converted to its glyoxylic acid and then derivatized with 2,4- dinitrophenylhydrazine to glyoxylate-2,4-dinitrophenylhydrazone before analysis [1]. Other methods of allantoin determination have included, colorimetry methods [2,3], reversed-phase high-performance liquid chromatography (HPLC) [4–8], HPLC with pre-column derivatization [9] and ion chromatography [10]. However, most of these methods include long and tedious sample preparation procedures when used with biofluids. The analysis of allantoin has been reviewed recently [11].

Uric acid has been shown to act as an antioxidant defence in humans against reactive oxygen species including free radicals [12–15]. Its oxidation by radical oxygen species will give various products including allantoin. In the absence of uricase, the appearance of allantoin in biofluids in man would therefore indicate free radical scavenging by uric acid. Allantoin has been determined in human urine, plasma or tissue extracts using HPLC [6–8,10] to estimate free radical generation in the body.

The ability of capillary electrophoresis (CE) to operate at low UV wavelengths should be ideal for

*Corresponding author.

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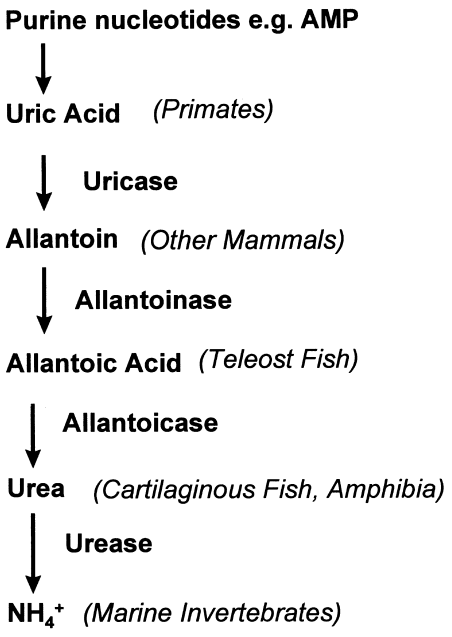

Fig. 1. Degradation of uric acid to ammonia via allantoin. Enzymes that catalyse the degradation of each product are shown in bold. Species that excrete at each product are shown in italics.

the determination of allantoin and its precursors. No work on the determination of allantoin in biological fluids such as urine has been reported using CE. The only reported analysis of allantoin by CE to date has been in cosmetics [16,17].

This paper presents results of our studies on the CE separation of allantoin from other endogenous compounds in urine from different animal species. The method involves minimal sample preparation and does not require derivatization. It is simple and fast. The method can also measure related metabolites such as uric acid.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulphate (SDS), sodium hydroxide, sodium tetraborate and all standards were obtained from Sigma–Aldrich (Poole, UK) and were of analytical grade.

2.2. Electrophoretic separation conditions

All experiments were carried out on a CE 2000 Instrument (TSP, Stone, UK) using fused-silica capillaries of 44 cm (effective length 37 cm)×75 μm I.D. (Composite Metal Services, Hallow, UK).

On-column fast-scanning multi-wavelength UV absorbance detection was employed. Spectral data of the migrating solutes was collected from 195 to 300 nm at 5-nm intervals (21 wavelengths) and analysed using SpectraSystem Software. Hydrodynamic injection mode for 4 s was used unless stated. New capillaries were conditioned for 30 min at 50°C with 0.4 *M* NaOH.

Capillary zone electrophoresis (CZE) buffer conditions were 30 m*M* sodium tetraborate, pH 10 unless stated. For micellar electrokinetic capillary chromatography (MECC), the running buffer was as for CZE but with the addition of 75 m*M* SDS. pH was adjusted before addition of the SDS. All stock running buffer solutions were stored at +4°C in between analyses.

Each day before analysis the capillary was prewashed with 0.4 *M* NaOH for 10 min at 40°C, then for 10 min with running buffer at 40°C and another 10 min of running buffer to bring the capillary temperature to 20°C. Prior to analysis the capillary was washed with the running buffer for 2.0 min at 20°C and then filled with buffer for another 2 min.

Capillary temperature of 20°C and a voltage of 20 kV was used for all separations throughout the study. Integration parameters for peak areas were carried out at 195 nm and corrected peak areas were always used for quantitation. At the end of each day the capillary was washed with deionised water at 40°C for 5 min, and left overnight in water.

2.3. Standard solutions preparation

Stock solutions of 10 m*M* of allantoin, xanthine, hypoxanthine, creatinine and uric acid standards were prepared separately by dissolving the appropriate amounts in a buffer of 15 m*M* sodium tetraborate, pH 8. Urea and allantoic acid were prepared at 100 m*M* and 40 m*M*, respectively. All standards were stored at +4°C. Standards were diluted as appropriate with water or buffer for analysis; concentrations used are indicated in the

text, tables and legends. For linearity studies allantoin concentrations ranging from 0.1–1.0 m*M*, were prepared and run six times at each concentration.

2.4. Sample preparation and spiking procedures

Dog, horse, rabbit, rat, human and mouse urines were stored at −55°C in 2-ml aliquots. Human urine was pooled from 10 healthy subjects. Daily, a fresh aliquot was thawed at room temperature as required and filtered through a 0.45-μm filter, before injection. For all animal urines dilution was 10-fold in 15 m*M* sodium tetraborate buffer, pH 8, and 2-fold for human urine sample. For recovery studies the diluted urines were spiked with allantoin to give a final concentration of 1 m*M*. Unless stated a buffer of 15 m*M* sodium tetraborate, pH 8 was used for diluting and preparing all samples, and referred to as just buffer in the text.

3. Experimental

3.1. CZE vs. MECC of catabolite products of purines

Allantoin, allantoic acid, hypoxanthine, xanthine and urea are all products in the purine metabolism pathway and are therefore likely to be found in the urine of different species. Creatinine and urea are useful marker peaks that should occur in all urines. Using a modification of a method previously developed for the separation of human urine endogenous compounds [18], the above seven analytes were analysed under CZE and MECC conditions. The electropherograms are compared in Fig. 2. It was found that allantoin and hypoxanthine co-migrated at pH 10, as did urea and creatinine and xanthine and uric acid under CZE conditions but were well resolved under MECC conditions. In CZE allantoic acid eluted after the hypoxanthine and allantoin peaks but using MECC it migrated before these two peaks.

3.2. Effect of pH on separating allantoin from other endogenous compounds

Urine being a complex mixture and containing a multitude of compounds, it is likely that some compounds will migrate as one peak. In order to ascertain that allantoin was eluting as one peak a study varying pH was carried out. The pH effect on the separation of allantoin from all other urinary endogenous was studied using human urine spiked with allantoin. Since allantoin is not a major catabolite in man, the changes in the migration of allantoin with the change in pH would be easier to monitor. In Fig. 3, the separation of urine at pH 9, 9.5 and 10 under MECC conditions is shown. Allantoin resolved well from all other endogenous compounds at all three pHs studied but the migration time shifted. At pH 9, it was observed that a peak between the peaks identified as hippuric acid and uric acid, did not completely resolve from both peaks. However at pH 9.5 and 10 the hippuric acid and uric acid peaks were resolved from this peak. No major differences in peak profiles was observed at these two pH values, except for the analysis time which increased by 1 min at pH 10. pH 9.5 was chosen for separation of the animal samples as this was rapid <6 min and gave a good separation of uric acid. Other peaks identified spectral analysis are shown on the electropherograms.

3.3. Allantoin and allantoic acid stability

Stability of allantoin and allantoic acid in solution was studied over eight days. Allantoin was dissolved in deionised water or buffer as a 2 m*M* solution stored at +4°C in between analysis. Allantoic acid was only dissolved in the buffer at a concentration of 10 m*M*.

After five days a small peak appeared before the allantoin dissolved in the buffer which was later identified as allantoic acid (Fig. 4a). The peak increased in size with increase in time. Fig. 4b shows the spectra of the allantoic acid obtained compared to spectra of allantoic acid. Migration time, spiking and peak purity of this peak correlated with that of standard allantoic acid. The calculated percentage degradation of allantoin to allantoic acid over two days was 10%.

3.4. Validation studies

The following allantoin concentration were used to

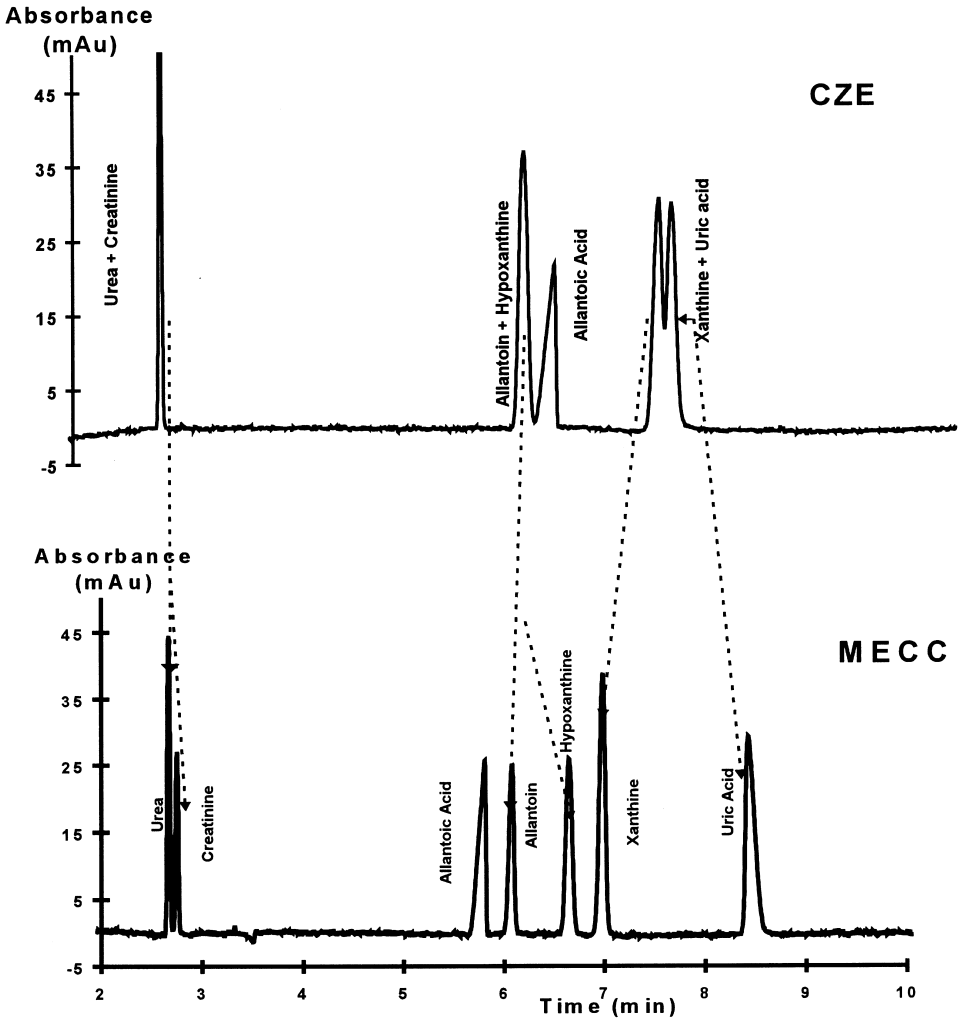

Fig. 2. Electropherograms showing the separation of seven standards, urea (100 m*M*), creatinine (1 m*M*), allantoic acid (4 m*M*), allantoin (1 m*M*), hypoxanthine (0.1 m*M*), xanthine (1 m*M*) and uric acid (1 m*M*) using CZE. Conditions: 30 m*M* sodium tetraborate, pH 10, 4 s hydrodynamic injection, 20°C, 20 kV and detection 195 nm and MECC conditions with addition of 75 m*M* SDS to the buffer.

construct a linear curve, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 m*M* dissolved in buffer. A calibration curve of the corrected peak area versus allantoin concentration was linear over the studied range. The equation of the line was $y=(88\,090\pm1899)x+(4691\pm1126)$ with $r^2=0.998$. The limit of detection was 40 μ*M* ($S/N=2$) using multi-wavelength detection or 5 μ*M* using single-wavelength detection at 195 nm. The corrected peak areas for the range of the concentrations used were reproducible with a relative standard deviation (R.S.D.) of 2 to 7%. The mean migration time of allantoin was 5.4 min.

Intra-assay variability of migration times for allantoin and allantoic acid was 2.6 and 3.3%, respectively and for corrected peak area it was 1.9 and 6%, respectively. Inter-assay variability of migration times over eight days was 4.6 and 5%, respectively and for corrected peak areas it was 10 and 24%, respectively.

In order to assess the accuracy of the developed

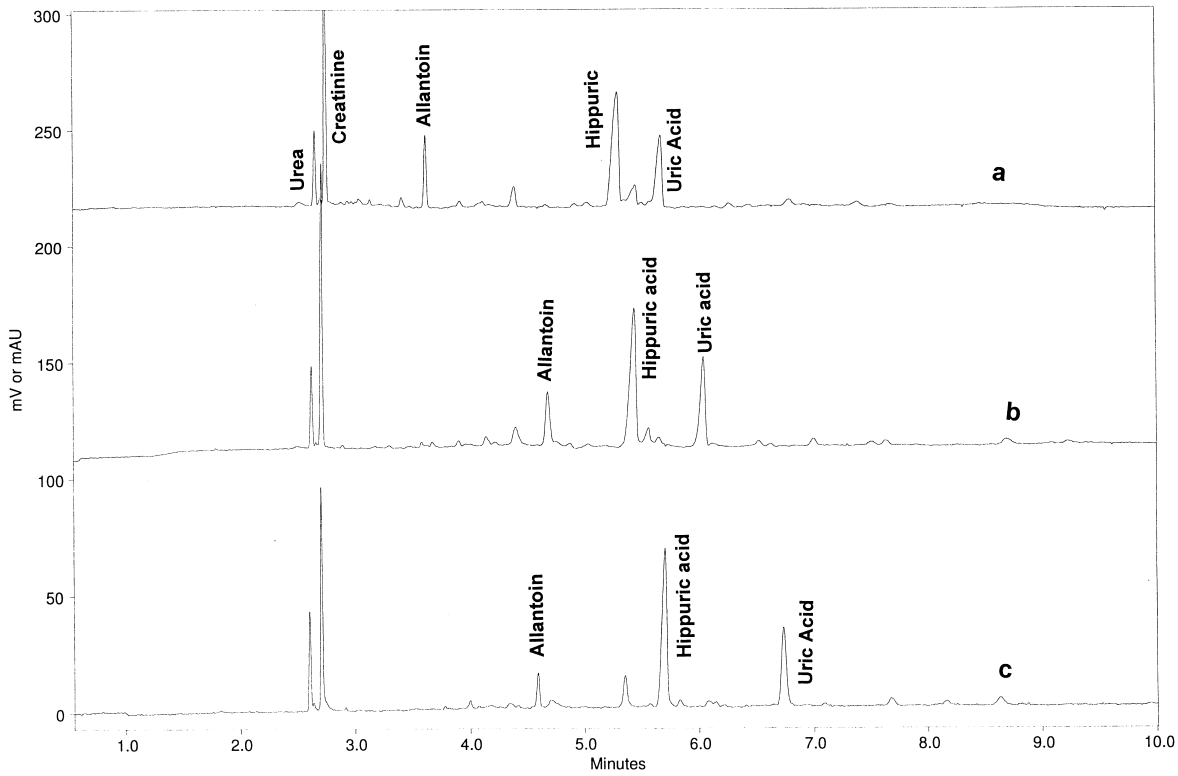

Fig. 3. Human urine spiked with allantoin analysed under MECC conditions as in Fig. 2, except for pH which was varied at (a) 9, (b) 9.5 and (c) 10.

method, known amounts of allantoin (1 m*M*) were spiked in dilute urines of dog, horse rat, human and rabbit as described in Section 2.4. Blank urines were prepared to calculate the recovery of allantoin in the spiked urines as described in Section 2.4. The overall allantoin recovery from the urine samples was calculated by comparing the corrected peak areas of blank urine and the spiked urine. Table 1 shows the recovery percentage of allantoin and their R.S.D.s in the urine samples studied.

3.5. Allantoin and other compounds in the urine samples

Following the above studies, the MECC conditions used to separate compounds in the animal and human urines were 30 m*M* sodium tetraborate, pH 9.5, 75 m*M* SDS, 20 kV, 20°C, 2 s hydrodynamic injection. Typical electropherograms are shown in Fig. 5a–f. Different profiles were observed between species, but major peaks observed in all urines migrated with similar migration time and are shown in the Figures. In Fig. 6a blank human urine spiked with allantoin (c) was compared with an electropherogram of standard allantoin (b). A small peak at 4.3 min, migrating where allantoin peak eluted in other animal urines, was observed. However spectral analysis of this peak showed that it was not allantoin.

4. Discussion

As illustrated in Fig. 2, MECC was ideal for separation of allantoin from the endogenous compounds in the urines and the migration time window was increased. Allantoin was very mobile with change in pH but was always well resolved from all other compounds. Depending on the pH other com-

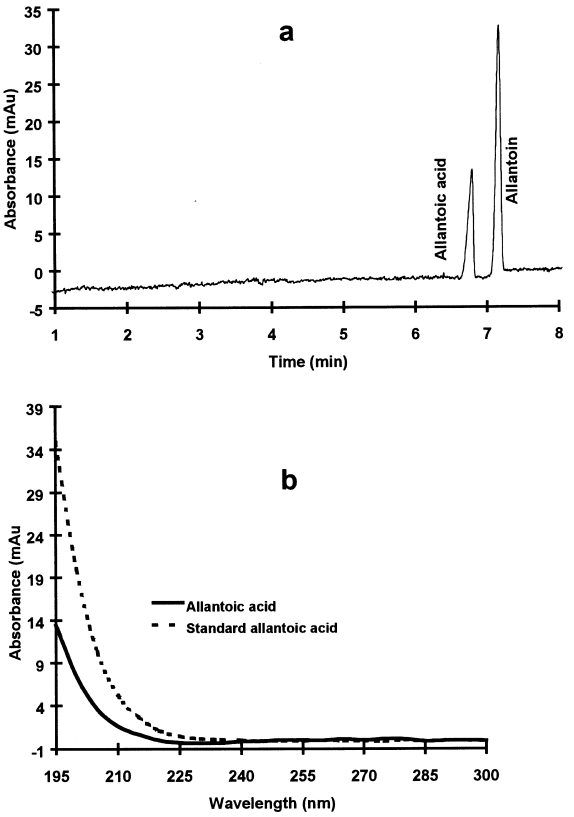

Fig. 4. (a) Electropherogram showing degradation of allantoin (5 m*M*) to allantoic acid stored at +4°C after three days. (b) Spectra of standard allantoic acid compared to the spectra of allantoic acid peak in (a). Conditions as in Fig. 2.

pounds resolved well while others co-migrated. Migration time of urea and creatinine peaks in Fig. 2 did not change with change in pH.

In the metabolic pathway of purine shown below Fig. 1, uric acid is degraded to ammonia through a series of oxidation reactions. Some, if not all, of these products are likely to be found in some urine samples. Using the MECC method uric acid and urea were simultaneously determined with allantoin.

Initially urine were dissolved in pH 10 buffer but it was observed that allantoin broke down to allantoic acid. These results were similar to those reported in previous work where allantoin degraded to allantoic acid if left to stand at room temperatures in an alkali environment [4]. Some standard compounds were insoluble in water at room temperature but dissolved in alkaline conditions e.g., uric acid. Preparation of all the standards were therefore carried out using a buffer of pH 8 to maintain the solution environment.

The animal urines were very concentrated relative to man, and required 5-fold dilution compared to human samples. However to get consistency in the sample and standards preparation, urine samples were diluted in a buffer of pH 8.

From the urine samples studied, it was noted that although allantoin is the major catabolite expected in mammalian species uric acid was also detected in most animal species. However in human urine only uric acid was observed. The MECC method developed was robust and could be applied to urine sample from any animal species.

Table 1
Recoveries of allantoin added to the spiked urine samples ($n=6$)

	Recovery (%)	R.S.D. (%)
Dog	101.5	8.90
Horse	100.4	5.65
Mouse	96	6.40
Rat	98	6.3
Rabbit	96.7	6.75
Human	84.7	4.08

Conditions: 30 m*M* sodium tetraborate, pH 9.5, 75 m*M* SDS, 20 kV, 20°C, 2.0 s hydrodynamic injection.

5. Conclusions

We have described a method for the simultaneous measurement of allantoin and uric acid in urine from different animal species including man using MECC. The MECC described here is simple, robust and fast with allantoin determined in <5 min. The final conditions were 30 m*M* sodium tetraborate, pH 9.5, 75 m*M* SDS, 20 kV and 20°C. Careful preparation and storage of allantoin standard solution was necessary for validation and quantification studies in the biofluids.

Allantoin was not detected in human urine. Therefore this method would be used to analyse biofluids from humans for the presence of allantoin to indicate oxidation status of uric acid and possibly a disease state.

Further experimental work is in progress for the optimisation of the developed method for separation

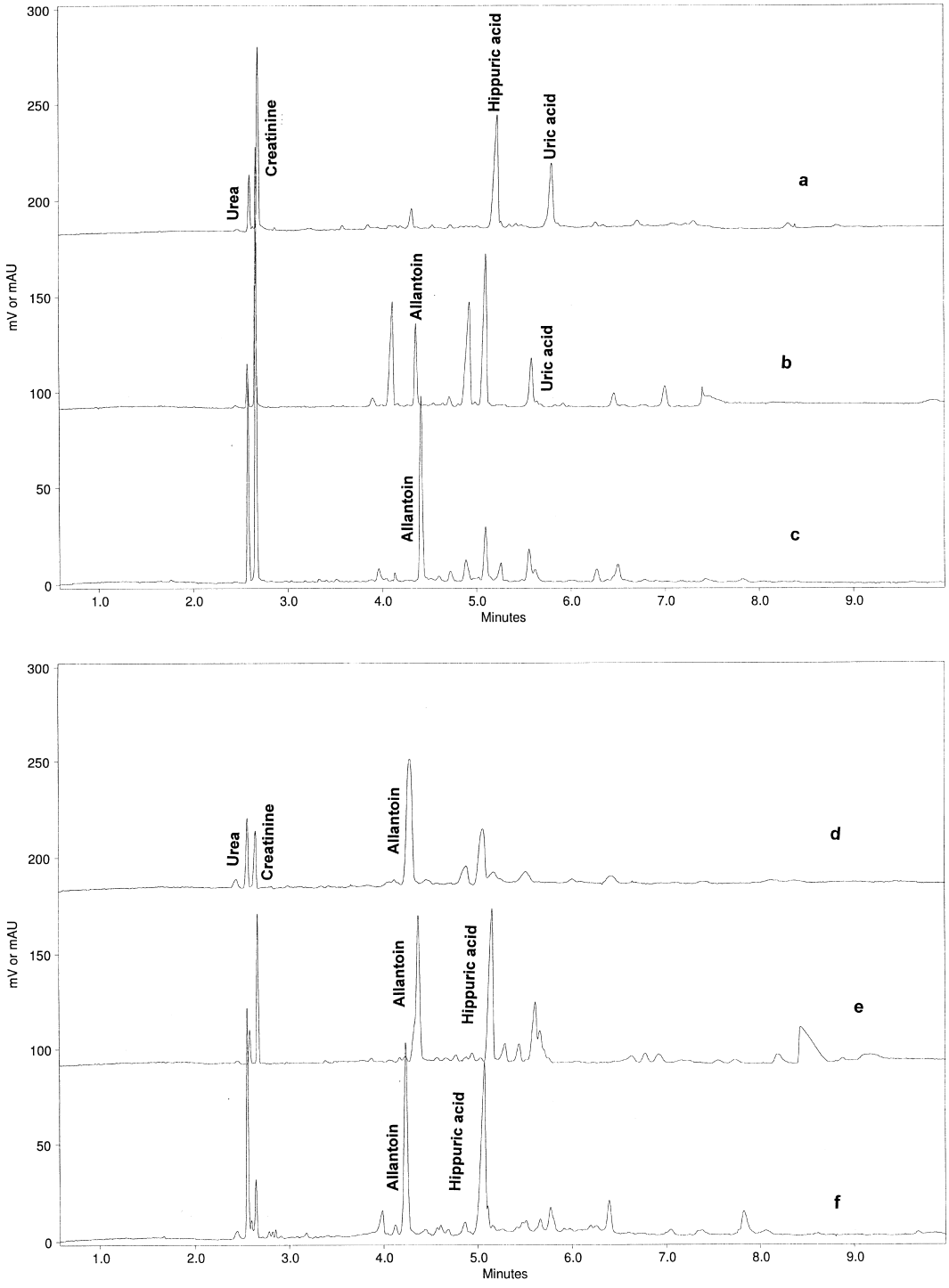

Fig. 5. Comparison of electropherograms of (a) human, (b) horse, (c) dog, (d) mouse, (e) rabbit and (f) rat urine showing the allantoin peak and other identified compounds. Conditions as in Fig. 2 with buffer pH at 9.5.

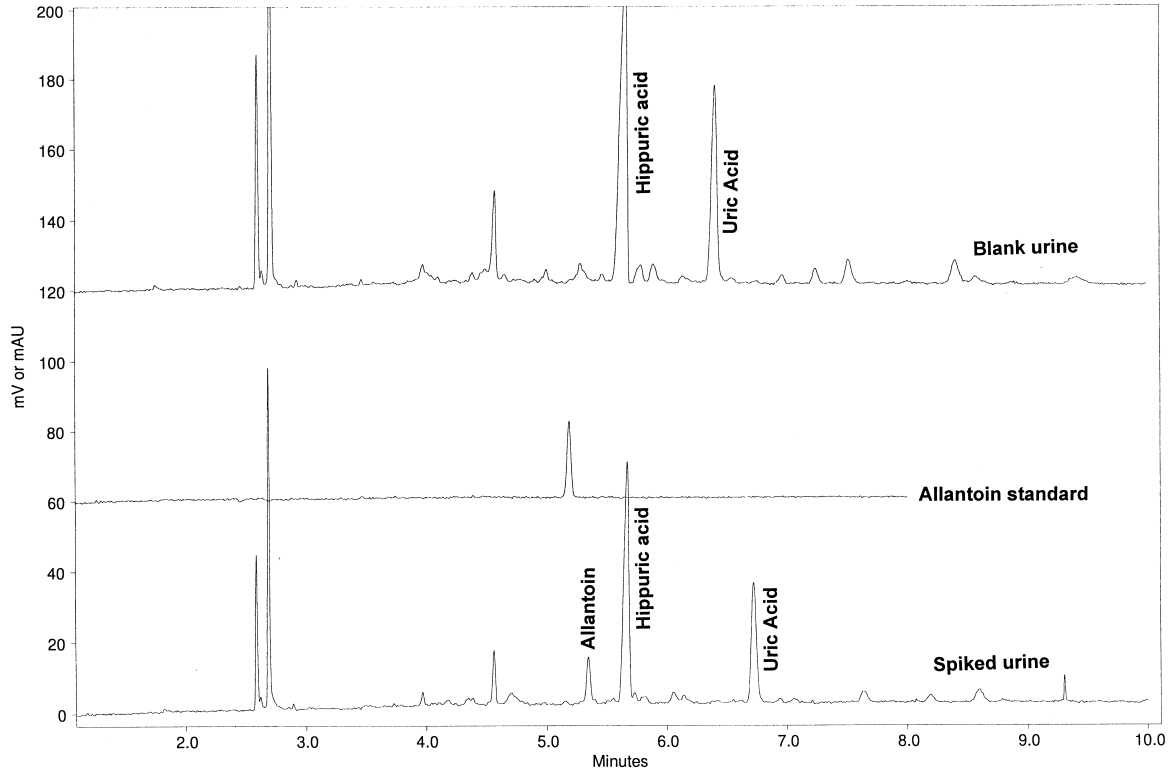

Fig. 6. Spiking of human urine with 1 m*M* allantoin. Conditions as in Fig. 5.

of the compounds and its modification for determination of metabolites in different biofluids such as plasma, and cerebrospinal fluid and their identification.

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