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Development of a capillary electrophoresis method for the determination of allopurinol and its active metabolite oxypurinol

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Abstract

A simple and sensitive capillary zone electrophoresis method with UV absorbance detection is described for the quantitation of allopurinol and its metabolite oxypurinol in aqueous solution. The influence of different parameters on migration times, peak symmetry, efficiency and resolution was systematically investigated; these parameters included the nature and concentration of the separation buffer, pH and applied voltage. A buffer consisting of 15 mM 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES) adjusted to pH 8.8 was found to provide a very efficient and stable electrophoretic system for the analysis of these compounds. The optimized method was validated with respect to precision, linearity, limits of detection and quantification, accuracy and robustness. The applicability of the assay was demonstrated by analyzing these compounds in serum and allopurinol in commercial pharmaceutical preparations.

Keywords: Allopurinol; Oxypurinol

1. Introduction

Allopurinol (ALP) and its mayor metabolite oxypurinol (OXP) are potent inhibitors of xanthine oxidase, the enzyme that converts hypoxanthine to xanthine, and xanthine to uric acid (Fig. 1). Allopurinol is commonly used in the treatment of chronic gout or of hyperuricaemia associated with leukaemia, radiotherapy, anti-neoplastic agents and treatment with diuretics [1].

Procedures capable of simultaneously detecting the primary drug (ALP) and its metabolite (OXP) in body fluids are of considerable interest for pharmacokinetic and clinical studies. Several assays for the determination of both compounds have been reported using high-performance liquid chromatography (HPLC) with either ultraviolet [2–9] or electrochemical detection [10,11], using ion exchange HPLC [2,3] and reversed phase HPLC [4–11]. Some of these methods have low limits of detection and quantitation, but others show various shortcomings. For example, the use of organic solvents (acetonitrile or methanol) [3] and excessively long elution times for allopurinol [2].

Capillary electrophoresis (CE) has gained a significant degree of acceptance in the analytical laboratory owing to its many advantageous features such as extremely high efficiency, high resolution, rapid analysis and low consumption of sample and reagents. However, CE methods proposed for the determination of ALP and OXP are scarce. Wang and co-workers [12] have reported a capillary zone electrophoresis (CZE) with end-column amperometric detection method using a running buffer composed of Na_2HPO_4/NaH_2PO_4 at pH 9.55 and detection potential at 1.20 V (versus Ag/AgCl electrode). Hempel and co-workers [13] have developed a CE assay with UV detection for the determination of ALP, OXP and other purine and products in urine with the aid of two running buffer formulations. In a first step, OXP was resolved by CZE using 60 mM sodium tetraborate at pH 8.7 as running buffer and, in a second step, micellar electrokinetic capillary electrophoresis (MEKC) with sodium dodecyl sulphate (80 mM) was used to resolve ALP. Despite the progress in CE separation of these two compounds, there is still room for improvement in the CE separation of allopurinol and oxypurinol. Accordingly, the aim of the present investigation was to optimize the CE conditions for the determination of both analytes. The effects of pH, type of buffer and its concentration and applied voltage on mobility, resolution, sensitivity and speed were carefully evaluated.

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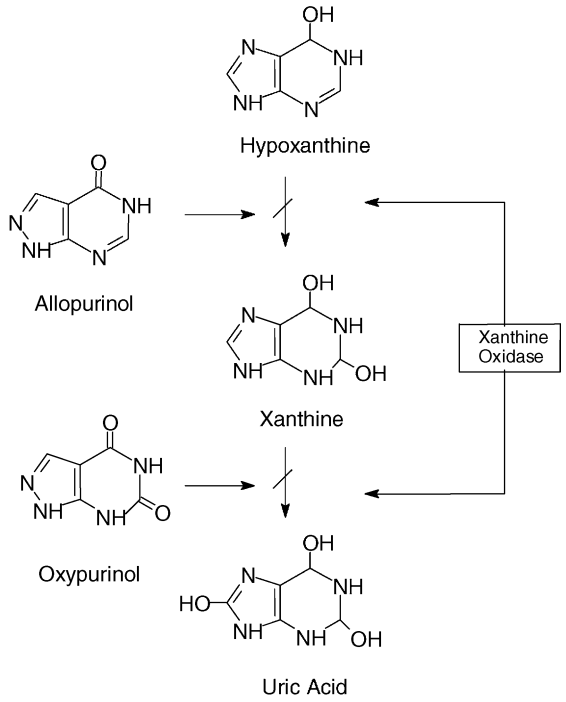

Fig. 1. Diagram showing the inhibitory effect of allopurinol and oxypurinol for the formation of uric acid.

The assay was validated by determining its accuracy, precision, linearity, specificity and robustness. The method has been successfully applied to the determination of ALP in pharmaceutical formulations and of both analytes in plasma.

2. Experimental

2.1. Reagents

ALP and OXP were purchased from Sigma (St. Louis, MO, USA). The organic solvents: acetone, acetonitrile, ethanol and methanol were of HPLC grade (Romil, Leoughborough, Leicestershire, UK). Mesityl oxide and sodium dodecyl sulphate were obtained from Sigma; other chemicals were of analytical grade. Ultrapure water from a Milli-Q plus system (Millipore Ibérica, Madrid, Spain) was used throughout. Borate, phosphate, HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid) and TRICINE (*N*-tris[hydroxymethyl]methylglycine) buffers were prepared by dissolving these reagents in ultrapure water and adjusting to the desired pH with sodium hydroxide or hydrochloric acid. All solutions were filtered through a 0.45 μm filter (Millisolve kit, Millipore), and then degassed by sonication and evacuation.

2.2. Apparatus

The CE equipment was purchased from Beckman Instruments (Palo Alto, CA) and consisted of Model P/ACE 5000 coupled to a diode array detector, a fluid-cooled column cartridge and an automatic injector. The apparatus was connected to a personal computer with a System Gold software (Beckman). Absorbance was monitored at 250 nm. Sample injection was carried out in a hydrodynamic mode during 5 s with a pressure of 0.5 p.s.i. (3.45 kPa). The voltage during the analysis was 15 kV (current: 10.9 μA) and the temperature thermostatized at 30 °C.

2.3. Electrophoretic procedure

All the experiments were carried out in uncoated fused-silica capillaries of 75 μm i.d., total length 57 cm (distance between injection and detection 50 cm) thermostatized at 30 °C. When a new capillary was used, the capillary was conditioned by first rinsing with 1 M sodium hydroxide for 10 min, then with 0.1 M sodium hydroxide for 10 min, followed by pure water for 10 min and finally with the running electrolyte for 10 min. To ensure reproducibility, the capillary was washed every day with 0.1 M sodium hydroxide for 3 min, followed by the running buffer for 10 min, and then equilibrated with the buffer for 2 min, while applying the separation voltage. Between each injection, the capillary was rinsed with fresh electrolyte solution (2 min). Rising for 2 min was sufficient to guarantee that the contents of the previous injection were eliminated and that the capillary was filled with fresh electrolyte. Unless otherwise stated 15 mM CHES buffer of pH 8.8 was used as the electrophoretic buffer. The pressure difference used for rinsing was always 0.5 p.s.i.

Electroosmotic flow was determined from the migration time of mesityl oxide or acetone, which were considered to be neutral throughout the entire sequence of buffers used. Electrophoretic mobilities, μ_{EP}, were calculated as the difference between the apparent mobility, μ_{app}, of each analyte and the mobility of neutral marker, μ_{EOF}, [14] using the equation:

$$\mu_{EP} = \mu_{app} - \mu_{EOF} = \frac{lL}{V}\left(\frac{1}{t_m} - \frac{1}{t_{EOF}}\right)$$

where l and L are the capillary length to the detector and the total capillary length, V is the applied voltage, and t_m and t_{EOF} are the migration time of the analyte and the neutral marker, respectively.

3. Results and discussion

3.1. Effect of buffer pH

Structures of allopurinol and oxypurinol are shown in Fig. 1. The ionization of the 1-NH group in the pyrazole moiety leads to the mono-anion form and further ionization of the 5-NH group in the pyridine moiety cause the formation of the di-anion form [15,16]. Hence, both compounds

can exit as neutral, mono-anion and di-anion species, depending on buffer pH.

It is interesting to note that the electrophoretic mobility of allopurinol and oxypurinol continuously decreased with increasing buffer pH. This suggests that the negative net charge of the drugs continuously increases up to the maximum value (net charge $= -2$), as is deduced from the fact that the first and second ionization constants are close to each other. Values of pH <7.5 could not be used because the baseline disturbance corresponding to the electroosmotic break-through time is very close to the peak of allopurinol. The separation was optimum in the pH range 8.5–9.5.

3.2. Effect of buffer nature and ionic strength

Five different aqueous buffers were tested: phosphate, borate, CHES, HEPES and TRICINE, all adjusted to the optimum pH range 8.5–9.5. The resolution between ALP and OXP peaks was achieved using any one of these buffers as electrophoretic electrolyte. However, only phosphate and CHES buffers yielded a good separation between the ALP peak and the electroosmotic break-through time of the buffer. Since CHES buffer at a pH of 8.8 provided the best results as regards stability of the base line, difference in the migration time of the analytes and peak symmetry, this buffer was selected for further studies.

The influence of CHES concentration on migration times and resolution was investigated. Migration and resolution increased when the concentration of CHES was increased. However, at 40 mM or higher concentrations, unstable base line and deformed peaks began to be observed. A 20 mM concentration of buffer was selected in order to reduce the analysis time while maintaining good resolution.

3.3. Optimization

When more than one variable is potentially important, it is difficult to obtain optimal conditions through the commonly used step-by-step optimization procedure. The experimental design offers an efficient route for identifying the conditions, yielding the best resolution and the shortest analysis time.

Three variables (buffer pH, applied voltage and buffer concentration) and two responses, corresponding to the resolution between ALP and OXP peaks (S_1) and the analysis time (last migrating compound, i.e. OXP) (S_2) were involved. The low and high levels of each variable were selected from the results obtained for the univariate method.

The results of the first set of experiments show that buffer pH and buffer concentration had a positive effect on S_1, but a negative effect on S_2. The applied voltage had a smaller effect and negative impact on S_1 and S_2. In the second set of experiments, the increase in the buffer pH had a small effect, being positive for S_1 and negative for S_2, which meant that the optimum parameter value was near. An increase in buffer pH still had a positive effect on S_1 (and negative for S_2) but a lower buffer concentration was not necessary. A final set of experiments was then carried out and the optimal conditions for S_1 (15 mM CHES buffer at pH 8.8 and 15 kV) and S_2 (15 mM CHES at pH 8.8 and 18 kV) were found. Since the optimal conditions predicted by the model for S_1 and S_2 were not completely the same, a compromise condition was obtained by balancing. Thus, 15 mM CHES buffer at pH 8.8 and an applied voltage of 15 kV were selected as the optimal conditions ($I \approx 10.9\,\mu$A).

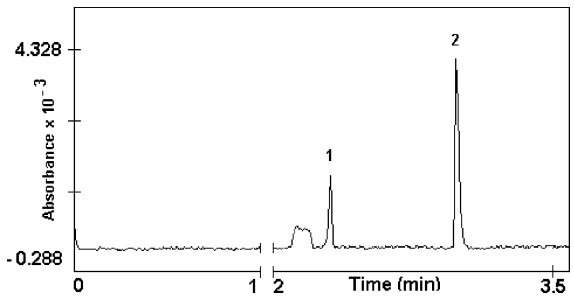

Fig. 2. Electropherograms obtained from a standard solution of allopurinol (2.2 μg/ml) and oxypurinol (5.2 μg/ml). The electrolyte was 15 mM CHES buffer, pH 8.8. The effective capillary length was 50 cm, the total length 57 cm and the applied voltage 15 kV; hydrodynamic injection was performed for 5 s at 3.45 kPa. Peaks: (1) allopurinol; (2) oxypurinol.

3.4. Method validation

Fig. 2 represents an electropherogram obtained under the conditions selected for the separation of ALP and OXP. Under these conditions, the two compounds are separated in less than 4 min and appear on the electropherogram as highly efficient and symmetrical peaks.

Validation of this method included assessment of stability of the solutions, specificity, linearity, detection and quantification limits, precision, accuracy and robustness.

3.4.1. Stability of the solutions

Although this test is often considered as a part of ruggedness of the procedure, it should be carried out at the beginning of the validation procedure because it conditions the validity of the data of the other tests.

The response factors of standard solutions were found to be unchanged for at least up to 12 days. Less than a 0.3% concentration difference was found between the solutions freshly prepared and those aged for 12 days. The solutions can, therefore, be used within this period without the results being affected.

3.4.2. Specificity

For CE methods, developing a separation involves demonstrating specificity. As the procedure described is for application to serum sample, the response of ALP and OXP in the test mixtures containing the two analytes and endogenous compounds of serum such as uric acid, hipoxanthine, xanthine and adenosine were studied. No significant electrophoretic interference was found. Due to the migration

time reproducibility, this CE method allowed the unambiguous discrimination of ALP and OXP in these samples.

3.4.3. Linearity

The assay was calibrated in the range of 0.68–96 μg/ml for ALP and 0.77–154 μg/ml for OXP (at least 10 samples covering the whole range of concentrations were used). External calibration was used because no improvement was observed when an internal standard was used. Each point of the calibration graph corresponded to the mean value from three independent peak area measurements. The linearity curves were defined by the following equations: $y = (0.0204 \pm 0.0001)x + (0.0104 \pm 0.0075)$, $r = 0.9996$ for ALP and $y = (0.0223 \pm 0.0001)x + (-0.0220 \pm 0.0116)$, $r = 0.9998$ for OXP, where y is the peak area of the analyte and x the drug concentration expressed in μg/ml.

3.4.4. Precision

The intra-day precision was tested with eleven repeated injections of two sample solutions containing the analytes at two concentration levels. The relative standard deviations (R.S.D.) for the peak area were 0.55 and 0.53% at the 7 μg/ml level and 0.32 and 0.48% at the 70 μg/ml level for ALP and OXP, respectively.

The inter-day precision of the method was studied by analyzing three identical samples, injected six times every day, on 5 consecutive days. The R.S.D. for the peak area were 0.99% for ALP and 0.96% for OXP.

The migration times were very reproducible. The intra- and inter-day precision, expressed as R.S.D., were 0.37 and 0.72% for ALP and 0.41 and 0.68% for OXP, respectively.

3.4.5. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was evaluated from five independent samples, which were spiked to produce a peak height for each of these compounds close to three times the base line noise [14]. The LOD was estimated by taking three times the standard deviation of the peak areas obtained from these solutions and calculating the corresponding concentration. The LOD obtained were 0.08 μg/ml for ALP and 0.12 μg/ml for OXP.

The LOQ is defined as the level at, or above, which the measurement precision is satisfactory for quantitative analysis. It was estimated by taking 10 times the standard deviation of the peak areas obtained from the five samples and subsequently calculating the corresponding concentration. The LOQ were 0.58 and 0.67 μg/ml for ALP and OXP, respectively.

3.4.6. Accuracy

The accuracy of the proposed method was tested with several synthetic mixtures containing both compounds in different proportions. ALP/OXP mixtures in the ratios from 1:10 to 10:1 were analyzed by the proposed CZE procedure. The results obtained were excellent because the recoveries ranged between 98.6 and 101.4%.

Table 1
Robustness of the analytical method upon variation of CE separation conditions

Parameter	Resolution
Voltage	
12	8.3
15	12.4
18	10.7
Buffer concentration (mM)	
10	12.5
15	12.4
20	14.8
Buffer pH	
9.0	15.1
9.5	12.4
10.0	9.6

3.4.7. Robustness

Robustness is defined as the capability of analytical procedure to remain unaffected by small but deliberate variations in the method parameters. Sufficient resolution was always obtained under all the separation conditions tested (Table 1), demonstrating sufficient robustness.

3.5. Applications

The CZE assay is characterized by long-term stability and reproducibility. More than 2000 analyses could be performed without having to replace the capillary. To demonstrate the usefulness of the procedure for the determination of ALP and OXP, pharmaceutical formulations and human serum were analyzed.

The serum samples were spiked with different quantities of both drugs, so that their concentrations were within the range 1–5 μg/ml for ALP and 4–8 μg/ml for OXP, which correspond to the reported peak plasma concentrations observed after a single oral dose of 300 mg of ALP [17]. The samples were deproteinized with acetonitrile and centrifuged (3 min at 1000 g). The liquid supernatant was used for the analysis following the general procedure.

Preliminary experiments to confirm the linearity and precision of the assay using spiked serum samples revealed that response was linearly dependent on concentration in the range of 0.7–12 μg/ml. The correlation coefficient of the regression lines was 0.999 or higher. The precision of the method was assessed by determining three concentrations within the range of 2–6 μg/ml in six independent series of samples. Table 2 summarizes the results obtained. Day-to-day precision data were also observed over a period of 5 working days; R.S.D. < 2.6% were always obtained.

A serum sample from a healthy female volunteer who had taken 300 mg of ALP was repeatedly analyzed. The results show a mean value of 1.96 μg/ml (S.D.: 0.03, $n = 6$) for ALP and 5.04 μg/ml (S.D.: 0.04, $n = 6$) for OXP. Fig. 3 shows typical electropherograms from the blank plasma and from the plasma containing the drugs. As can be seen by

Table 2
Analytical recovery and precision of the CZE method for serum samples

Compound	Amount added (μg/ml)	Amount found (μg/ml)	R.S.D. (%)	Recovery (%)
ALP	2	2.1 ± 0.05	1.18	105
	3	3.01 ± 0.04	0.66	100.3
	4	4.07 ± 0.03	0.53	101.7
OXP	3	2.77 ± 0.03	0.54	92.3
	4	3.97 ± 0.18	2.60	99.2
	6	6.01 ± 0.13	1.08	100.1

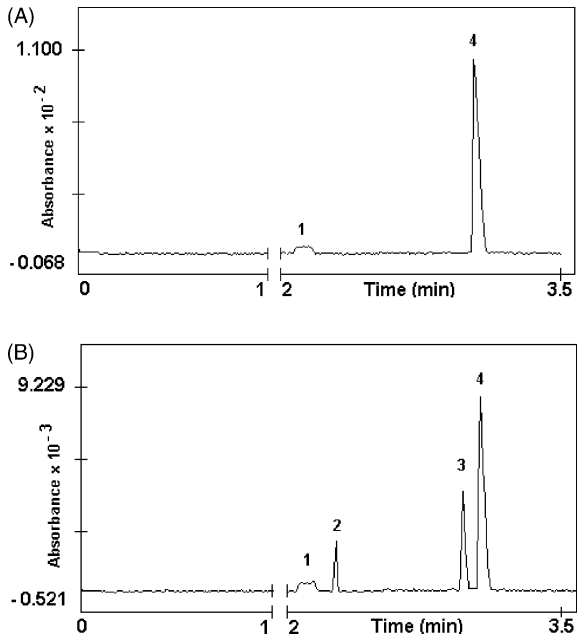

Fig. 3. Electropherograms of: (A) blank plasma, and (B) from a volunteer under allopurinol therapy (see text). Electrophoretic conditions were same as for Fig. 2. Peaks: (1) xanthine; (2) allopurinol; (3) oxypurinol; (4) uric acid.

Table 3
Determination of ALP in pharmaceutical formulations

Sample (supplier)[a]	Nominal content (mg per tablet)	ALP found (mg per tablet)	
		CZE method[b]	Reference method[c]
Zyloric (Faes)	100	101.3 ± 1.5	99.5
Facilit (Fides)	100	101.6 ± 2	99.2
Acifugan (Lacer)	100	102.8 ± 3.8	100.7

[a] Composition: Zyloric: ALP, 100 mg; lactose monohydrate, starch, povidone and magnesium stearate. Facilit: ALP, 100 mg; Aerosil 2000, magnesium stearate, talcum, Avicel PH 101. Acifugan: ALP, 100 mg; lactose, starch, polyvinylpyrrolidone, sodium laurylsulfate, sorbic acid, magnesium stearate, colloidal silica, talcum, sodium carboxymethyl cellulose, sodium citrate, propylene glycol, titanium dioxide, polysorbate 80, eugradit L, c.s.

[b] Mean of five determinations ± S.D.

[c] UV spectrophotometric method. Values are the mean of two determinations.

comparison of both electropherograms, there are no endogenous interfering peaks at the migration times corresponding to ALP and OXP.

The method developed was also used to quantify ALP in three commercial formulations. The results obtained are summarized in Table 3.

4. Conclusion

A simple, rapid, efficient and reliable method for CZE separation and determination of ALP and OXP has been developed using 15 mM CHES buffer adjusted at pH 8.8 as electrophoretic electrolyte. The method was validated with regard to precision, specificity, linearity, limits of detection and quantification and robustness. The usefulness of this method is demonstrated by the excellent results obtained in the determination of ALP and OXP in human serum and ALP in different pharmaceutical formulations.

When this method was compared to the other CE methods [12,13], it can be seen that its LOD is generally as good as the previously reported methods. However, this assay is simpler than that proposed by Hempel and co-workers [13] and more reliable than that proposed by Wang and co-workers [12] because it uses absorbance detection rather than home-made amperometric detection.

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References

[1] J.G. Hardaman, L.E. Limbird (Eds.), The Pharmacological Basis of Therapeuties (Godman & Gilman's), 10th ed., McGraw-Hill, New York, 2001, p. 721.
[2] E. Endele, G. Lettenbaucer, J. Chromatogr. 115 (1975) 228.
[3] M. Brown, J. Bye, J. Chromatogr. 143 (1977) 195.
[4] G.J. Putterman, B. Shaikh, M.R. Hallmark, C.G. Sawyer, C.V. Hixson, F. Perini, Anal. Biochem. 98 (1979) 18.
[5] S.L. Chang, W.G. Kramer, J. Chromatogr. 181 (1980) 286.
[6] H. Miyazaki, Y. Matsunaga, K. Yoshida, S. Arakawa, M. Hashimoto, J. Chromatogr. Biomed. Appl. 25 (1983) 75.
[7] R. Boulien, C. Bory, P. Baltassat, C. Gonnet, J. Chromatogr. Biomed. Appl. 307 (1984) 469.
[8] C.T. Hung, A.R. Zoest, D.G. Perrier, J. Liq. Chromatogr. 9 (1986) 2471.
[9] K. Safranow, Z. Machoy, K. Ciechanowski, Anal. Biochem. 286 (2000) 224.
[10] F. Palmisano, E. Desimoni, P.G. Zambonin, J. Chromatogr. Biomed. Appl. 306 (1984) 205.
[11] E.J. Eisenberg, P. Conzentino, G.G. Liversidge, K.C. Cundy, J. Chromatogr. Biomed. Appl. 95 (1990) 65.

[12] X. Sun, W. Cao, X. Bai, X. Yang, E. Wang, Anal. Chim. Acta 442 (2001) 121.
[13] T. Wessel, C. Lanvers, S. Fruend, G. Hempel, J. Chromatogr. A 894 (2000) 157.
[14] D.R. Baker, Capillary Electrophoresis, Wiley, New York, 1995.
[15] F. Bergman, A. Frank, Z. Neiman, J. Chem. Soc. Perkin I (1979) 2795.
[16] A.C. Moffat, Clarke's Isolation and Identification of Drugs, The Pharmaceutical Press, London, 1986.
[17] H. Breithaupt, M. Tittel, Eur. J. Chim. Pharmacol. 22 (1982) 77.