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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 813–822
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Application of capillary electrophoresis with pH-mediated sample stacking to analysis of coumarin metabolites in microsomal incubations

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Received 24 April 2002; received in revised form 28 February 2003; accepted 3 March 2003

Abstract

A sensitive method for the analysis of metabolites of coumarin by capillary electrophoresis (CE), incorporating pH-mediated sample stacking, was developed. The analytes were detected in phosphate buffer (pH 7.5; 25 mM), the matrix of the microsomal incubations. Detection was by direct UV absorbance. The three metabolites studied were 7-hydroxycoumarin (7-OHC), 4-hydroxycoumarin (4-OHC) and 2-hydroxyphenylacetic acid (HPAA), and the limits of detection of the analytes were 0.1, 0.5 and 0.3 μM, respectively. The developed method was then applied to microsomal incubations of coumarin. Male Cynomologus monkey microsomes were used in the study and 7-OHC was detected in the incubation mixture.

Keywords: CE; pH-mediated stacking; Coumarin; Microsomal incubations

1. Introduction

The study of in-vitro metabolism reactions can provide valuable information about the biotransformation of drugs. Information such as kinetic factors and inhibition constants, as well as information regarding the activity of the specific enzyme involved can be determined from metabolic data. Coumarin is an ideal probe drug for carrying out these metabolism studies, as it is a substrate specifically for cytochrome P450 2A6 and is a simple organic substance that is metabolized at several sites, forming a variety of products. Thus it serves as a model for the metabolism of more complicated structures [1].

Traditionally, HPLC has been used to monitor the metabolism of coumarin [2–5], although more

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0731-7085/03/$ - see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/S0731-7085(03)00184-5

recent research has employed capillary electrophoresis (CE) [6–8]. There are several advantages to choosing CE over HPLC for the analysis of metabolic reactions. As the BGE can be changed easily between runs if necessary, metabolic mixtures can be injected directly onto the capillary without the need for prior protein removal, a step which is essential in HPLC to avoid fouling the typically expensive columns. Furthermore, the high voltage applied in CE effectively terminates the metabolic reaction without the need for a separate termination step. CE is thus a convenient tool for the analysis of metabolic mixtures, as it permits separations with the need for only minimal sample clean up prior to analysis.

Detection for CE is most commonly performed by UV absorbance. UV absorbance is a popular choice for detection as it is simple and cost effective when compared with other modes of detection such as electrochemical detection and mass spectroscopy. CE with UV detection however, suffers from poor concentration sensitivity when compared with other methods, with typical detection limits in the parts per million range. This poor sensitivity results from both the small sample volumes (picoliters to nanoliters) introduced onto the capillary and the short optical pathlength for detection. The low concentration sensitivity of CE–UV is particularly evident when performing analyses of metabolic mixtures. Minor metabolites are typically formed in low concentrations and may fall outside the limit of detection of a conventional CE–UV method. A further difficulty with the analysis of physiological samples is the typically high ionic strength of the matrix. This can lead to 'destacking' effects, which are evidenced by band-broadening and can further affect detection limits. Band-broadening also occurs when there is incompatibility between the sample matrix and the BGE. To combat this, samples can be prepared in the BGE, though this is not always a feasible option.

Both on-column and off-column concentration techniques can be employed to improve detection limits for low concentration analytes. Off-column techniques include liquid–liquid extraction and solid-phase extraction techniques, as well as on-line coupling of an LC to the CE instrumentation [9]. These techniques generally provide much improved sensitivity but at the cost of increased analysis times. On-column techniques include field amplification stacking and pH-mediated sample stacking. Field amplification is the most commonly used on-column technique, and involves preparing the sample in a low-conductivity matrix, typically H_2O, before injection. Although field amplification stacking can provide effective on-column concentration, it is limited to samples of lower ionic strength than the BGE. Physiological samples, therefore, must undergo extraction, or else be significantly diluted before they can be injected.

pH-mediated sample stacking is an on-capillary concentration technique that can be used to overcome these limitations for physiological samples. When base-stacking is performed for the analysis of anions, the electrokinetic sample injection is immediately followed by an electrokinetic injection of sodium hydroxide. In order to effectively introduce anionic compounds by electrokinetic injection, it is necessary to reverse the intrinsic direction of the electroosmotic flow (EOF). This is achieved by adding an appropriate surfactant to the BGE. The base titrates the BGE, resulting in the formation of a high electric field zone. This causes the analytes of interest, in this case the anions, to stack at the interface of this low conductivity zone and the BGE, resulting in an increased response for low concentration analytes. Samples prepared in Ringers solution have previously been analyzed by this technique [10–12], achieving sensitivity enhancements of up to 66-fold [10]. This research aimed to perform pH-mediated sample stacking of metabolites in an incubation matrix consisting of a phosphate buffer and to apply this to a study of the metabolism of coumarin by the Cytochrome P450 2A6 isoform in Cynomologus monkey microsomes.

2. Experimental

2.1. Reagents and analytes

7-Hydroxycoumarin (7-OHC), 4-hydroxycoumarin (4-OHC), coumarin, methylamine hydro-

chloride, tetradecyltrimethyl-ammonium bromide (TTAB), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) and D-glucose-6-phosphate (dipotassium salt) were purchased from Sigma (St. Louis, MO). 2-hydroxyphenylacetic acid (HPAA), potassium dihydrogenphosphate and ammonium hydroxide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Glucose-6-phosphate dehydrogenase (from yeast) was purchased from ICN (Costa Mesa, CA). Male Cynomologus monkey microsomes were purchased from In Vitro Technologies Inc. (Baltimore, MA). All other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of solutions

The background electrolyte was prepared from 50 mM methylamine and adjusted to pH 10.7 by addition of 1 M ammonium hydroxide. In order to reverse the EOF, TTAB was added to the BGE at a concentration of 0.5 mM [13]. 0.1 M stock solutions of 7-OHC, 4-OHC and HPAA were prepared in pure methanol. These standards were then diluted to 10 μM in phosphate buffer (pH 7.5; 25 mM) and in the BGE to prepare standards for injection. The standards were prepared in the BGE to provide a control, to compare the peaks of the stacked standard with a case where no band-broadening would be expected to occur. $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase solutions were prepared in phosphate buffer (pH 7.5; 25 mM). Nanopure water (18.2 Ω) was used throughout the study.

2.3. Microsomal incubations

The incubation method followed was similar to that described by Deasy et al. [7] The incubations were carried out by mixing 195 μl of coumarin standard (50 μg/ml), 15 μl of $NADP^+$ (20 mM), 10 μl of glucose-6-phosphate (50 mg/ml) and 5 μl of glucose-6-phosphate dehydrogenase (4 U/ml) in a glass vial. The concentration of the microsomes used was 20 mg/ml and was indicated by the supplier. These concentrations are higher than those required to maintain maximum metabolic rates under the conditions chosen. The incubations were initiated by the addition of 25 μl of microsomes to the incubation vial. Cynomologus monkey microsomes were chosen for the study for their similarity in metabolic profile of 7-OHC to human microsomes [8]. Male monkey microsomes were used as data from the supplier indicated a higher level of coumarin 7-hydroxylation in the male of the species. The vial was maintained at 37 °C in a heating block. Aliquots were taken from the mixture at timed intervals over the period of the incubation and centrifuged for 10 min at 12 400 r.p.m. to remove excess protein. This protein should not harm the capillary but its removal reduces the risk of clogging of the capillary during the analysis and also allows for more sensitive detection of the analytes of interest. A series of controls were carried out for the incubations by substituting, respectively, the coumarin, $NADP^+$ and microsomes in the incubation mixture with 25 mM phosphate buffer, pH 7.5. Metabolites were identified by comparison of the incubation mixtures with the control incubations and by the spiking of the incubation mix with a standard solution of the suspected metabolite.

2.4. Capillary electrophoresis separation

Separations were carried out on a Beckman P/ACE MDQ instrument (Fullerton, CA). The capillary was fused silica, 50 μm x 61.2 cm (50 cm to detector). The capillary was initially primed by sequential flushing at 30 °C with MeOH (5 min), 1 M HCl (5 min), H_2O, (2 min) 0.1 M NaOH (5 min), H_2O (2 min) and the BGE (7 min). Between runs the capillary was flushed at 50 p.s.i. for 1 min with 5 mM SDS—0.1 M NaOH (50:50, v/v), 1 min with 0.1 M NaOH and 1 min with the BGE. Samples were introduced onto the capillary by a 45 s electrokinetic injection at −10 kV, followed by a 90 s NaOH injection at −10 kV for sample stacking. The concentration of the NaOH was 50 mM, to match the concentration of the BGE [14]. Separation was carried out at −20 kV with a rise time of 0.17 min. Detection was by UV absorbance at 214 nm.

3. Results and discussion

3.1. Development of the separation conditions

CE has previously been used to monitor the in vitro metabolism of coumarin, both in humans and in other species [6–8]. The electrophoretic methods used were based on the separation of the primary metabolite, 7-OHC, from the metabolic mixture. Limits of detection of 7-OHC for these studies were 6.17 μm (1 μg/ml) [6,7]. 7-OHC, however, is not the only known metabolite of coumarin in man. Various other metabolites, such as 4-OHC and ring-opening metabolites, were not included in these original studies. Our research aimed to expand the separation of metabolites of coumarin in microsomal incubations with increased sensitivity. Methylamine was chosen as the BGE as it gave a full separation of the three analytes of interest in under 7 min and was compatible with the pH-mediated stacking technique. Stacking injection parameters were optimized by varying the lengths of both sample and base injection until optimum conditions were reached (Tables 1 and 2). As the aim of the research was to develop a more sensitive method for the determination of metabolites, the optimal conditions were taken to be those which yielded the greatest peak heights. The greatest peak heights for all three analytes were achieved with a 45 s sample injection followed by a 90 s injection of the base. Separation of the three analytes under optimal conditions is shown in Fig. 1.

3.2. Evaluation of the stacking method

In order to evaluate the stacking method, separations were carried out as for the stacking method, but without the base injection. The injection time for these unstacked samples was also optimized (Table 3). Standards were also prepared in the background electrolyte for comparison (Table 4). From this data (Tables 1–4) it can be seen that the electropherograms obtained using pH-mediated sample stacking showed both greater peak heights and increased efficiency over the unstacked samples. For example, a 5 s injection of sample using the pH-mediated stacking method resulted in peak heights for 7-OHC of over double the peak heights of unstacked samples prepared in BGE and over triple those obtained for unstacked samples prepared in phosphate buffer. Longer injection times could also be utilized with the stacking method before signifi-

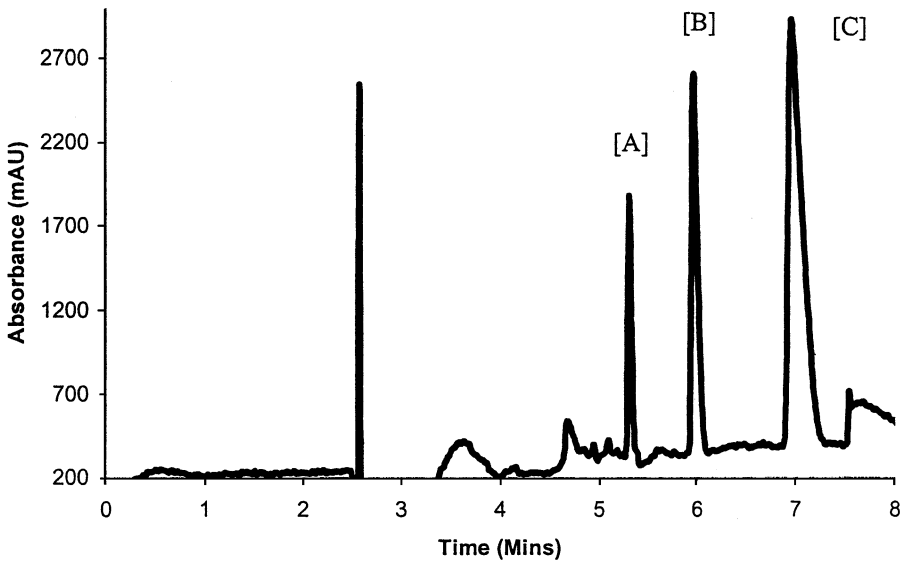

Fig. 1. Standard 10 μm mixture of (A) hydroxyphenylacetic acid (B) 7-hydroxycoumarin and (C) 4-hydroxycoumarin. Separation conditions were as described in Section 2.4.

Table 1
Optimisation of sample base injection ratio

Base inj. time (s)	HPAA peak height (mAU)	HPAA efficiency N(/1000)	7-OHC peak height (mAU)	7-OHC efficiency N(/1000)	4-OHC peak height (mAU)	4-OHC efficiency N(/1000)
45	1058±247	69±26	1791±323	79±38	1980±324	13±2
55	1462±33	112±7	2073±89	63±15	2298±87	13±2
65	1655±97	117±12	2169±74	62±16	2331±179	13±2
75	1705±185	107±6	2068±176	44±0.3	2443±139	13±2
85	1673±171	108±18	2225±84	51±8	2523±116	13±2
90	1963±127	106±13	2416±189	48±5	2607±444	11±1
95	1865±179	112±8	2332±92	49±2	2560±102	11±0.4
99	1954±216	114±17	2232±20	50±3	2315±243	10±1

Standards were 10 μM prepared in phosphate (pH 7.5; 25 mM). All sample injections were 45 s.

Table 2
Optimisation of sample injection time

Sample inj. time (s)	HPAA peak height (mAU)	HPAA efficiency N (/1000)	7-OHC peak height (mAU)	7-OHC efficiency N (/1000)	4-OHC peak height (mAU)	4-OHC efficiency N (/1000)
5	276±6	90±1	378±9	49±2	338±11	14±0.3
10	462±8	69±2	578±9	45±0.2	638±10	17± 0.6
15	558±6	77±2	729±16	52±3	697±13	14±3
20	811±13	73±3	1201±30	49±1	1166±9	14±2
25	1053±56	72±4	1399±14	45±3	1728±43	16±3
30	1194±9	72±5	1698±17	43±3	1957±22	16±3
35	1308±18	71±4	1819±71	42±2	2045±70	17±2
40	1449±97	66±2	2171±166	39±1	2248±104	17±1
45	1963±128	106±13	2416±189	48±5	2607±444	11±1
50	1641±18	65±2	2437±15	40±2	2482±78	17±0.3

Standards were 10 μm prepared in phosphate buffer (pH 7.5; 25 mM). All base injection times were double the respective sample injection time.

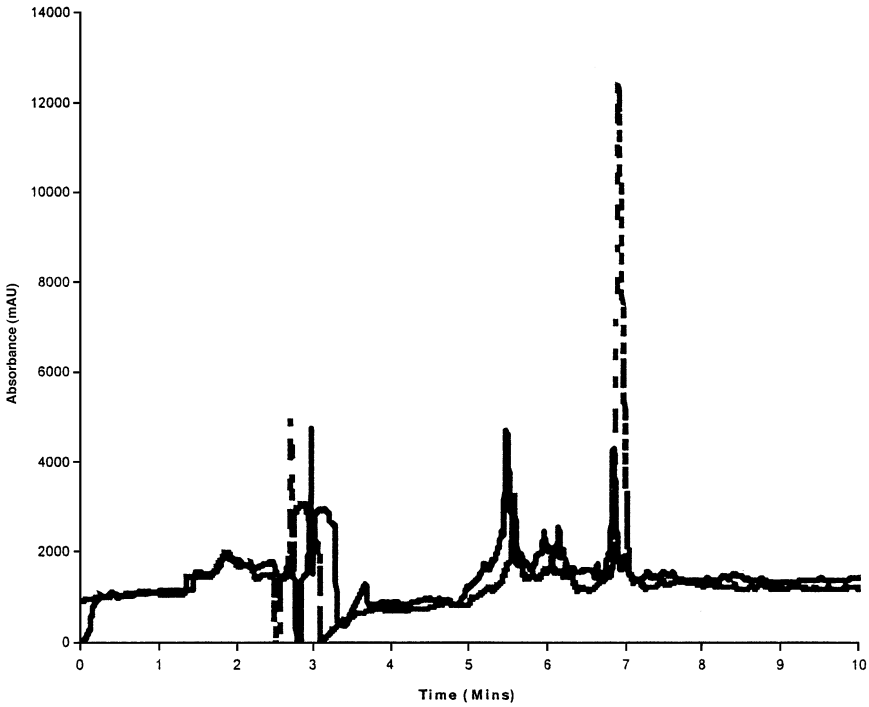

Fig. 2. Electropherogram of microsomal incubation of coumarin (—) and incubation of coumarin spiked with 7-OHC (----). Separation conditions were as described in Section 2.4.

cant loss of efficiency was observed. This is particularly evident in Table 4 where it can be seen that the efficiency for 7-OHC decreases dramatically after the 5 s injection. This can be attributed to the increased peak width for the analyte as the capillary is overloaded with sample.

3.3. Limits of detection

The limits of detection of the analytes, based on a signal to noise ratio of three were 0.1 μm for 7-OHC, 0.5 μm for HPAA and 0.3 μm for 4-OHC. This represents a 60-fold improvement in detection limits for 7-OHC by CE over previously published methods [6,7]. The linear ranges were 0.1–350 μm; 1–250 μm and 0.5–350 μm, respectively. Results were based on mean values of three runs and the R^2 values of the three calibrations were: 0.991, 0.992 and 0.965.

3.4. Microsomal incubations

Once the optimal conditions had been established, the method was applied to the microsomal incubation of coumarin. 7-OHC is the primary metabolite of coumarin in Cynomologus monkeys, with ring-opening metabolites and 3-hydroxycoumarin being formed to a lesser extent [15]. 7-OHC was detected in the microsomal incubations performed. The amount of 7-OHC formed was then calculated by comparison with a calibration curve. The calibration curves were based on 7-OHC peak area and calibration curves were performed on the day in which the assay was carried out in order to eliminate any day-to-day variations. No 4-OHC or HPAA were detected. Fig. 2 shows an electropherogram after a 90 min microsomal incubation of coumarin. Figs. 3 and 4 are electropherograms of control incubations, after 90 min carried out without microsomes (Fig. 3) and without $NADP^+$

Table 3
Peak data obtained for unstacked samples

Sample inj. time (s)	HPAA peak (mAU)	HPAA efficiency N (/ 1000)	7-OHC peak height (mAU)	7-OHC efficiency N (/ 1000)	4-OHC peak height (mAU)	4-OHC efficiency N (/ 1000)
5	29±24	29±6	113±8	32±7	84±25	22±9
10	60±7	8±1	167±11	11±2	154±13	6±0.9
15	62±1	4±0.3	145±11	7±0.7	165±22	3±0.4
20	97±32	5±3	189±34	3±0.3	215±7	2±0.4
45	116±26	3±0.7	249±33	3±0.1	248±17	2±0.1

Standards were 10 μm prepared in phosphate buffer (pH 7.5; 25 mM). Separations were performed as for the stacking experiments with the exception that no base injection was performed.

Table 4
Peak data obtained for samples prepared in BGE

Sample inj. time (s)	HPAA peak (mAU)	HPAA efficiency N (/ 1000)	7-OHC peak height (mAU)	7-OHC efficiency N (/ 1000)	4-OHC peak height (mAU)	4-OHC efficiency N (/ 1000)
5	99±7	84±16	167±23	65±10	154±20	37±14
10	140±8	33±9	266±7	38±4	288±52	22±1
15	143±13	14±4	300±10	19±4	395±16	18±2
20	131±9	16±4	286±19	25±6	373±25	19±2
45	143±25	19±4	298±18	26±5	385±27	18±0.4

Standards were 10 μm prepared in the BGE. Separations were performed as for the stacking experiments with the exception that no base injection was performed.

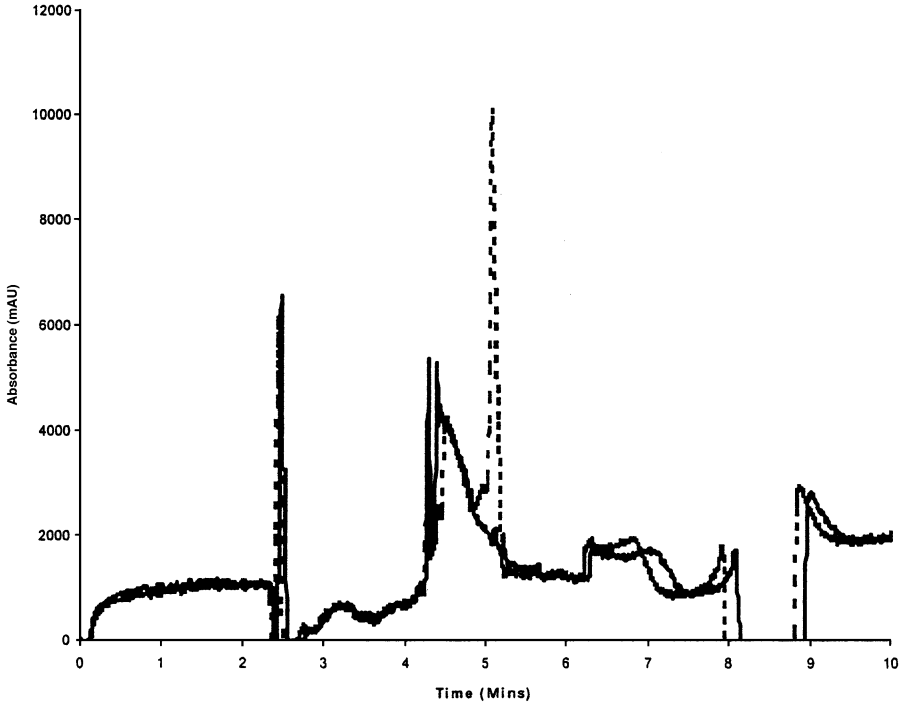

Fig. 3. Electropherogram of control incubation of coumarin carried out without microsomes (—) and control incubation without microsomes spiked with 7-OHC (----). Separation conditions were as described in Section 2.4.

(Fig. 4). Both of these incubations were spiked with 7-OHC to show its relative migration.

3.5. Production of 7-hydroxycoumarin

A plot of 7-OHC concentration produced (nmol per mg of protein) over time is shown in Fig. 5. For clarity, the time is plotted as the time at which the aliquot was taken from the incubation vial, although the incubation would proceed whilst the sample was being prepared. This would account for the appearance of 7-OHC in the sample taken at 0 min. The formation of 7-OHC proceeded in a fairly linear manner over the first 45 min, reaching a plateau as the activity of the microsomes decreased. At 45 min the activity of the microsomes was 438.4 pmol/mg/min. Previous research has quoted microsomal activity for Cynomologus monkeys as 616.8 pmol/mg/min [8] and 556.9 pmol/mg/min [15]. The activity of the microsomes at 90 min was calculated to be 223 pmol/mg protein/min. This is in agreement with characterization information provided by the microsome suppliers.

4. Conclusions

pH-mediated sample stacking was found to be easily applicable to the analysis of microsomal incubations of coumarin, providing a sensitive method for the determination of metabolites with minimal sample handling. The extent of coumarin 7-hydroxylation determined for Cynomologus monkey microsomes was found to be in agreement with previous research.

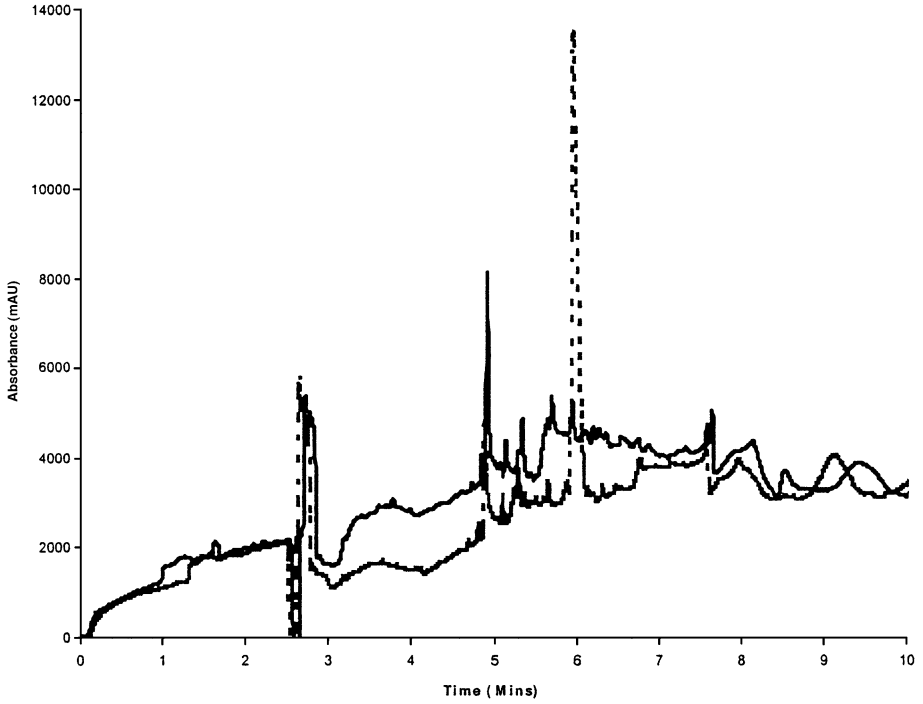

Fig. 4. Electropherogram of control incubation of coumarin carried out without microsomes (—) and control incubation without microsomes spiked with 7-OHC (----). Separation conditions were as described in Section 2.4.

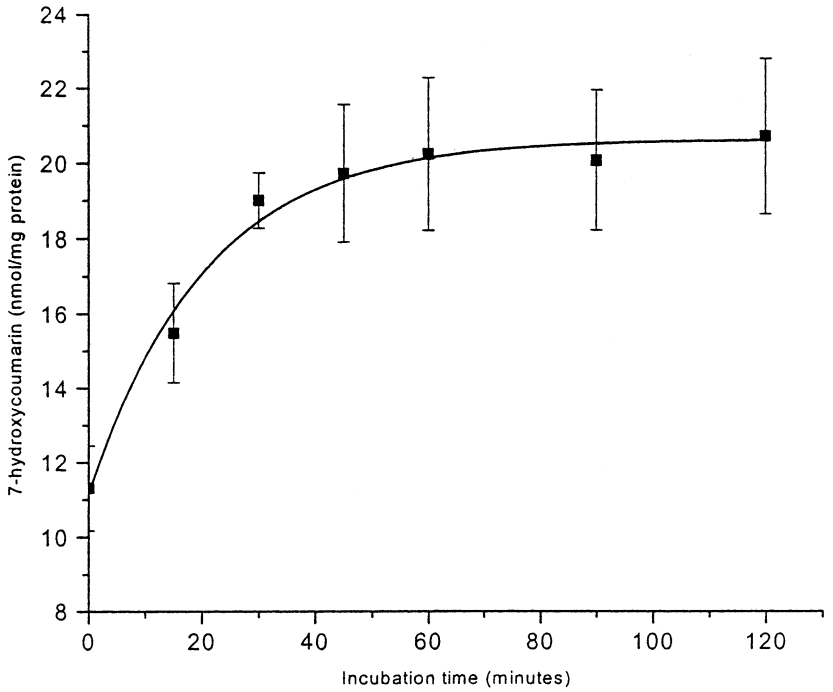

Fig. 5. Plot of 7-hydroxycoumarin (nmol/mg protein) ± standard deviation ($n = 3$) versus time for Cynomologus monkey microsomes.

Acknowledgements

The authors thank Enterprise Ireland for provision of a basic research grant (Number SC/98/265) and the N.I.H. for partial funding of this research.

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