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Analysis of aliphatic dicarboxylic acids in pharmaceuticals and cosmetics by liquid chromatography (HPLC) with fluorescence detection*

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Abstract: 2-Bromoacetyl-6-methoxynaphthalene has been found to be a useful prechromatographic fluorescent labelling reagent for the analysis of dicarboxylic acids. The derivatization reaction of azelaic acid and meglutol with this reagent yieded stable and highly fluorescent diesters which could be analysed by reversed-phase HPLC with fluorescence detection. According to the nature of the sample, the derivatization reaction could be carried out in acetonitrile or in an aqueous micellar system. The proposed methods proved to be suitable for the quality control of various complex pharmaceutical and cosmetic formulations of the azelaic acid and meglutol.

Keywords: *Reversed-phase liquid chromatography; dicarboxylic acids analysis; derivatization with 2-bromoacetyl-6-methoxynaphthalene; fluorimetric detection.*

Introduction

A great variety of carboxylic acids (fatty acids, bile acids etc.) is of biopharmaceutical importance, but the analysis of these acids often presents problems because of the poor detectability due to the absence of a strong chromophore and fluorophore. When polar carboxylic acids have to be analysed, high-performance liquid chromatography (HPLC) with UV and fluorescence detection, in combination with precolumn chemical derivatization, constitutes a convenient approach to overcome the problem [1, 2]. For the determination of dicarboxylic acids various reagents have been described, such as 4-bromomethyl-7-methoxycoumarin (BrMMC) [3], *o*-(4-nitrobenzyl)-N,N′-(diisopropyl)isourea (4-NBDI) [4], 4-bromophenacylbromide [4, 5], phenacyl bromide [6, 7] or a tosylate derivatizing agent such as [2-(1-naphthyl)-ethyl tosylate] [8]. Some of these methods, however, are time consuming and yield mono- and di-esters.

Recently, 2-bromoacetyl-6-methoxynaphthalene (Br-AMN) has been proposed by workers in this department as a useful fluorescent labelling reagent for the HPLC analysis of fatty acids and bile acids [9–11].

In the present study the applicability of Br-AMN to the HPLC analysis of the dicarboxylic acids azelaic acid (a keratolytic and anticomedogenic agent) and meglutol (an hypolipidaemic drug) was evaluated, in view of the current need of selective methods for their determination in pharmaceuticals and cosmetics.

The derivatization reaction was performed following two different procedures: in an aqueous micellar system; and in acetonitrile in the presence of triethylamine. The second method resulted in higher reaction yields whereas the first method allowed direct derivatization to be performed in an aqueous medium.

The procedures were successfully applied to the HPLC–fluorescence analysis of azelaic acid and meglutol in commercial pharmaceutical or cosmetic formulations.

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Experimental

Materials

Azelaic acid (nonanedioic acid) was obtained from the Sigma Chemical Company (St Louis, MO, USA). Meglutol (3-hydroxy-3-methylpentanedioic acid)), valproic acid (2-propylpentanoic acid), caproic acid (n-hexanoic acid), tetrahexylammonium bromide (THxABr) were obtained from Fluka (Buchs, Switzerland); triethylamine (TEA) and other chemicals were from Farmitalia C. Erba (Italy). For chromatography, acetonitrile, tetrahydrofuran and methanol were HPLC grade (Mallinkrodt, USA) and deionized double-distilled water was used. All other chemicals were of analytical-reagent grade. The reagent 2-bromoacetyl-6-methoxynaphthalene (Br-AMN) and the naphthacyl esters of valproic acid [9] and n-hexanoic acid [11] (used as internal standards) were prepared as previously described.

Apparatus

The liquid chromatograph comprised a Varian 2010 pump and a Varian 2070 fluorescence spectrophotometer, operating at an emission wavelength at 460 nm with an excitation wavelength of 300 nm, connected to a personal computer IBM XT-PC. The JCL6000 chromatography data system was used. Manual injections were carried out using a Rheodyne model 7125 injector with a 50-μl loop.

The solvents were degassed on line with a degasser ERC-3312 Erma (Tokyo, Japan). IR spectra were recorded in a Nujol mull on a Perkin-Elmer 298 IR spectrophotometer. UV spectra were recorded on a Jasco Uvidec 610 double-beam spectrophotometer.

^{1}H NMR spectra were recorded on a Varian EM 390 spectrometer at 90 MHZ and on a Varian Gemini spectrometer at 300 MHZ using tetramethylsilane as internal standard.

Sonarex Super RK 102 (35KHz) (Bandelin, Berlin, Germany) equipment with thermostatically controlled heating (30–80°C) was used for ultrasonication.

Synthesis of the naphthacyl diesters

The dicarboxylic acid (0.26 mmol) in about 6 ml of acetonitrile was treated with 1.06 mmole of the reagent, 2-bromoacetyl-6-methoxynaphthalene, in the presence of 0.3 ml of triethylamine for 30 min at 75°C. After cooling, the reaction mixture was diluted with 30 ml of water and then extracted with diethyl ether (3 × 10 ml). The combined extracts were washed with 5% (w/v) sodium bicarbonate solution and with water (3 × 10 ml), dried over anhydrous sodium sulphate and then evaporated *in vacuo*. The residue was purified by crystallization from ethanol to give a white compound which was found to be homogeneous by TLC using ethyl acetate–petroleum spirit (7:3, v/v). UV detection was at 254 and 366 nm.

6-Methoxynaphthacyldiester of azelaic acid. M.p. 135–136°C. IR (cm^{-1}):1735 (CO ester), 1690 (ketone), 1620, 1265, 1160, 1020, 900, 850. UV (ethanol): λ_{max} = 311 nm (ϵ 1.88 × 10^4). ^{1}H NMR (DMSO): δ 1.30–1.40 (m, 3 × 2H, C-CH_2-C), 1.57–1.68 (m, 2 × 2H, C-CH_2-C), 2.43–2.52 (t, 2 × 2H, CH_2-COO), 3.94 (s, 2 × 3H, OCH_3), 5.58 (s, 2 × 2H, COO-CH_2-CO), 7.2–8.65 (m, 12H, Ar).

6-Methoxynaphthacyldiester of meglutol. M.p. 108–110°C. IR (cm^{-1}): 1740 (CO ester), 1680 (CO ketone), 1625, 1270, 1180, 1025, 905. UV(ethanol): λ_{max} = 312 nm (ϵ = 2.82 × 10^4). ^{1}H NMR (DMSO): δ 1.44 (s, 3H, CH_3), 2.75–2.93 (q, 2 × 2H, C-CH_2-COO), 3.92(s, 2 × 3H, OCH_3), 5.0 (s, 1H, OH), 5.58 (s, 2 × 2H, COO-CH_2-CO), 7.25–8.65 (m, 12H, Ar).

Stock solutions

Solutions of the reagent (Br-AMN) (4.2 mg ml^{-1}) were prepared in acetone (Method A) or in acetonitrile (Method B) and were found to be stable for 2 weeks at 4°C. The analytical solutions were prepared in: water–methanol (8:2, v/v) (azelaic acid) or water–methanol (9:1, v/v) (meglutol) for the derivatization method A; and in acetonitrile for method B. Solutions of the internal standards (Table 1), 6-methoxynaphthacylesters of valproic acid and n-hexanoic acid, were prepared in acetonitrile (Method A) or in the mobile phase (Method B). Tetrahexylammonium bromide (THxABr) solution (20 mM) was prepared in aqueous 100 mM phosphate buffer (pH 7.0); 1% (w/v) triethylamine (TEA) solution was prepared in acetonitrile.

Derivatization procedure

Method A. To 0.2 ml of the carboxylic acid solution, 0.15 ml of 20 mM tetrahexylammonium bromide (THxABr) in 100 mM phosphate buffer (pH 7.0) and 0.1 ml of the

Table 1
Data for the calibration graphs (n = 6) obtained by the HPLC — fluorescence method for azelaic acid and meglutol derivatized with Br-AMN

Compound	Internal standard	Slope (±SD)	Intercept (±SD)	Correlation coefficient	Concentration range (nmol ml^{-1})
Azelaic acid	Valproic acid naphthacylester				
Method A	20 μg ml^{-1}	0.0125 (0.0002)	0.0063 (0.002)	0.9985	15.90–63.60
Method B	40 μg ml^{-1}	0.0083 (0.0002)	0.0075 (0.0025)	0.9970	49.20–262.90
Meglutol	n-hexanoic acid naphthacylester				
Method A	7.5 μg ml^{-1}	0.0042 (0.0001)	0.0410 (0.0015)	0.9990	15.90–206.70
Method B	24 μg ml^{-1}	0.0132 (0.0001)	−0.1400 (0.0350)	0.9990	32.74–204.60

reagent solution (4.2 mg ml^{-1}) in acetone were added. The derivatization reaction was allowed to proceed under stirring for 33 min at 70°C (azelaic acid) or 43 min at 65°C (meglutol). Then 0.15 ml of the appropriate internal standard solution was added; the reaction mixture was ultrasonicated at room temperature for 1 min and a 50-μl aliquot of the resulting clear solution was injected into the chromatograph.

Method B. An appropriate volume (0.1–0.2 ml) of the dicarboxylic acid solution in acetonitrile was reacted with 0.1 ml of the reagent solution (4.2 mg ml^{-1}) in acetonitrile, in the presence of 0.1 ml of 1% TEA solution, into a micro-reaction vessel (3.0 ml) at 40°C for 40 min. The reaction mixture was then evaporated to dryness with a stream of nitrogen. The residue was dissolved in 0.15 ml of the appropriate internal standard solution and 0.45 ml of the mobile phase with ultrasonication at room temperature for 1 min; a 50-μl aliquot was injected into the chromatograph.

Chromatographic conditions

The HPLC separations were performed at 35°C on a Hypersil 5 ODS (250 × 4.6 mm i.d.) stainless steel column under isocratic conditions. For routine analyses, a mobile phase of quaternary mixtures A–B, where A = acetonitrile–methanol–tetrahydrofuran (55:40:5, v/v/v) and B = water, was used. The analysis of azelaic acid was carried out using: mixture A–B (70:30, v/v) at a flow-rate of 1.2 ml min^{-1} for method A; and mixture A–B (68:32, v/v) at a flow-rate of 1.6 ml min^{-1} for method B. The analysis of meglutol was carried out using mixture A–B (65:35, v/v) at a flow-rate of 1.2 ml min^{-1} for method A and (70:30, v/v) at a flow-rate of 1,6 ml min^{-1} for method B.

Calibration graphs

Method A. Standard solutions of azelaic acid (15.9–63.60 nmol ml^{-1}) and meglutol (15.9–206.7 nmol ml^{-1}) were prepared in water–methanol (80:20, v/v) and water–methanol (90:10, v/v), respectively.

Method B. Standard solutions of azelaic acid (49.19–262.90 nmol ml^{-1}) and meglutol (32.74–204.60 nmol ml^{-1}) were prepared in acetonitrile.

An appropriate volume (0.1–0.2 ml) of the carboxylic acid standard solution was subjected to the appropriate derivatization procedure and 0.15 ml of the internal standard solution (Table 1) was added.

The peak-height ratio of the analyte to internal standard was plotted against the corresponding acid concentration to obtain the calibration graphs.

Analysis of pharmaceutical and cosmetic formulations

Sample preparation. Azelaic acid. An amount of the commercial preparation (pharmaceutical ointment, cosmetic lotion and cleansing lotion) equivalent to about 15 mg of azelaic acid was dissolved in 100 ml of methanol and an aliquot of the solutions was further diluted (2–10) with water. For analysis of a cosmetic compressed powder, a sample equivalent to about 15 mg of azelaic acid was powdered and treated with 100 ml of methanol by ultrasonication for 10 min and centrifugation for 20 min at 4000 rpm. The supernatant was filtered and an aliquot of the resulting clear solution was diluted (2–10) with water.

For the ointment, sample solutions were also prepared by direct dissolution in acetonitrile to give a final drug concentration of 18 μg ml^{-1}.

Meglutol. Five tablets were powdered and an amount equivalent to about 100 mg of the drug was treated with 10 ml of water. The suspension obtained was diluted to 100 ml with methanol and subjected to magnetic stirring for 10 min. After filtration, an aliquot of the clear solution was further diluted (1–10) with water.

Assay procedure. A 0.2-ml aliquot of sample solution was subjected to the derivatization procedure with the reagent (Br-AMN) using method A for the aqueous sample solution and method B for the solution in acetonitrile. The determination of the drug content in each sample was performed by comparison with an appropriate standard solution.

Results and Discussion

Derivatization reaction

The derivatization of azelaic acid and meglutol with Br-AMN was first carried out on a preparative scale and the reaction products were isolated and characterized. The IR (absence of the carboxylic bands), and NMR spectra confirmed that AMN diesters were obtained. For analytical applications, the derivatization reaction was studied in micellar aqueous medium (Method A) and in acetonitrile (Method B).

For the derivatization in an aqueous medium tetrahexylammonium bromide (THxABr) was selected as ionic micellar agent, according to previous studies [11–13]. The derivatization of azelaic and meglutol required high temperatures (33 min at 70°C and 40 min at 65°C, respectively) with magnetic stirring to reach a plateau (Fig. 1). Lower temperatures were inadequate whereas at higher temperatures there was an increase in decomposition. Under these conditions esterification was imcomplete (yield was about 70 and 20%, for azelaic acid and meglutol, respectively) but was found to be suitable for the HPLC determination of the dicarboxylic acids in commercial formulations. If necessary for meglutol the reaction yield can be remarkably increased by using more drastic conditions (70°C for 60 min).

In order to optimize the derivatization system, the reaction was carried out also in an organic solvent (acetonitrile) using 1% TEA. At 40°C, with magnetic stirring, the derivatization reaction of azelaic acid was found to be complete after 25 min (Fig. 2). Similar results

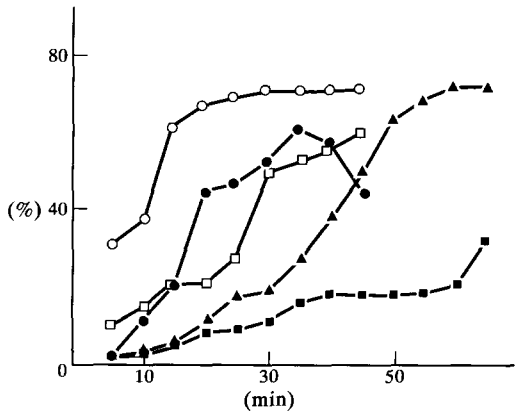

Figure 1
Influence of temperature on the derivatization of azelaic acid and meglutol with Br-AMN (Method A). Reaction of azelaic acid at 70°C with continuous magnetic stirring (○) and at 70°C (□) with ultrasonication for 3 min. Reaction of meglutol at 65°C (■), 70°C (▲) and 80°C (●) with continuous magnetic stirring. % = percentage yield of the reaction.

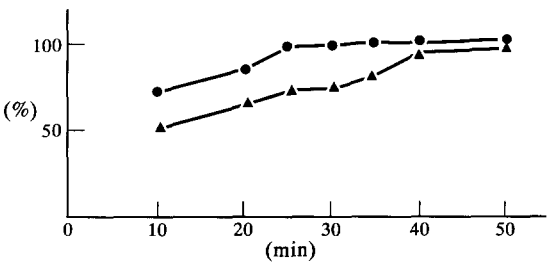

Figure 2
Influence of temperature on the derivatization of azelaic acid with Br-AMN (Method B). Reaction at ambient temperature (▲) and at 40°C (●) with continuous magnetic stirring.

were obtained for meglutol; the reaction yield increased to reach a plateau after 30 min at 40°C. Higher temperatures did not offer significant advantages. Under these conditions the derivatization reaction on azelaic acid was found to be essentially quantitative by comparison with an authentic specimen of azelaic acid naphthacylester; for meglutol the yield was 75% by comparison with meglutol naphthacylester.

Chromatography

Chromatographic separations were carried out under isocratic conditions on a reversed-phase column, Hypersil 5 ODS. As a result of previous experience [9–11] quaternary mixtures of acetonitrile, methanol, tetrahydrofuran and water were used for dicarboxylic acid analyses. For routine analyses a mobile phase

of the mixture A–B, where A = acetonitrile–methanol–tetrahydrofuran (55:40:5, v/v/v) and B = water, was found to be appropriate. The A/B ratios were suitably adjusted in order to obtain the separation of the various degradation products, which varied according to the derivatization method used. Under the chosen conditions these products did not interfere with the analysis, although method B (derivatization in acetonitrile) appears to be advantageous giving less degradation and allowing a more rapid analysis. Typical chromatograms are illustrated in Figs 3 and 4. It is likely that minor quantities of monoesters are also obtained but, owing to their higher hydrophilicity, these compounds, did not interfere with the analysis eluting at lower retention times close to the solvent front.

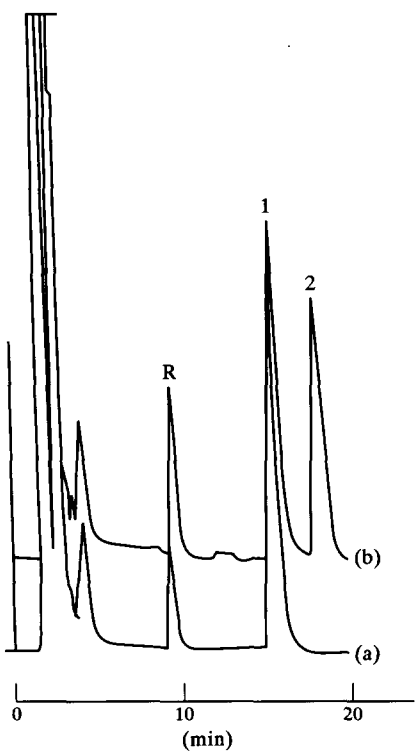

Figure 3
Representative HPLC chromatogram obtained at 35°C from: (a) Br-AMN reagent under reaction conditions; (b) derivatized azelaic acid (Method A). Peaks: 1, the internal standard (Table 1); 2, azelaic acid; and R = reagent peak. The internal standard was added at the end of the derivatization reaction. Column: 5-μm Hypersil 5 ODS (250 × 4.6 mm i.d.). Mobile phase: mixture A–B (70:30, v/v), where A = acetonitrile–methanol–tetrahydrofuran (55:40:5, v/v/v) and B = water, at a flow-rate of 1.2 ml min^{-1}. Fluorescence detection: λ_{em} = 460 nm; λ_{exc} = 300 nm. Attenuation 16.

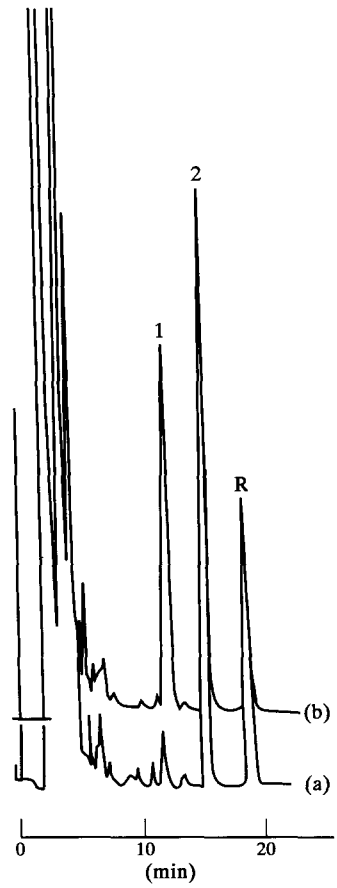

Figure 4
Representative HPLC chromatogram at 35°C of: (a) Br-AMN under reaction conditions; (b) derivatized meglutol (Method A). Peaks: 1, meglutol; 2, the internal standard (Table 1); and R = reagent peak. The internal standard was added at the end of the derivatization reaction. Column: as in Fig. 3. Mobile phase:mixture A–B (65:35, v/v), where A = acetonitrile–methanol–tetrahydrofuran (55:40:5, v/v/v) and B = water, at a flow-rate of 1.2 ml min^{-1}. Detection as in Fig. 3. Attenuation 16.

Analysis of pharmaceutical and cosmetic formulations

Azelaic acid is applied as a 15 of 20% cream to the lesions of malignant melanoma; it has also been given by mouth [14]. Azelaic acid has also been used in the treatment of acne. For its analysis few HPLC methods have been developed [3, 5–8] and these have limited applications in the analysis of pharmaceutical and cosmetic formulations [5, 6]; on the other hand, interest in the development of new topical delivery system for azelaic acid [6, 15] calls for suitable analytical tools.

Meglutol is used as a hypolipidaemic agent [14]; HPLC methods [4, 16–18] have been developed for the analysis of meglutol in human urine.

Table 2
Results for the HPLC determination of azelaic acid and meglutol in pharmaceutical and cosmetic formulations

Drug	Formulation*	Found†	RSD%
Azelaic acid	Ointment (Method A)	101.05	2.3
	(Method B)	100.10	0.85
	Compressed powder‡	99.17	2.1
	Cleansing lotion‡	99.20	1.6
	Lotion‡	99.98	2.4
Meglutol	Tablets	98.00	2.1

*Other ingredients. Ointment: fatty acids polyoxyethylene esters; cetylstearyl octanoate; glycerides and waxes mixtures; glycerol; propylene glycol; benzoic acid; purified water. Compressed powder: Hybrid powder (titanium dioxide-polyamide); N-lauryl L-lysine. Cleansing lotion: cocoylamide propylbetaine, dilauryl(7) OE; sodium citrate. Lotion: burdock (dry substance); Mimosa tenuiflora (dry substance); pyridoxine. Tablets: polyvinyl pyrrolidone; silica; magnesium stearate; talc; hydroxypropylmethylcellulose; titanium dioxide; polyethylene glycol; E110.

†Mean of five determinations and expressed as a percentage of the claimed content.

‡Cosmetic preparation.

The above cited drugs were, therefore, subjected to the described HPLC method involving pre-chromatographic derivatization with Br-AMN.

Under the described chromatographic conditions a linear relationship between peak-height ratio (analyte to internal standard) and analyte concentration (nmol ml^{-1}) were found for each drug (Table 1) using both derivatization methods, A (aqueous medium) and B (acetonitrile).

The precision of the methods was satisfactory as indicated by the relative standard deviations, obtained from replicate ($n = 8$) analyses (derivatization and HPLC separation) of a single standard solution (50 nmol ml^{-1}) of azelaic acid (Method A, RSD = 2.0%; Method B, RSD = 2.06%), meglutol (Method A, RSD = 2.9%; Method B, RSD = 2.55%).

For the analysis of the commercial formulations of the cited drugs, derivatization Method A was preferred because it offers the opportunity of directly analysing aqueous analytical samples, without solid phase extraction (SPE) procedures to transfer the analytes into acetonitrile. Lower derivatization yields were obtained but the sensitivity was not a problem in these applications and the precision was comparable to that of Method B. Derivatization in acetonitrile (Method B) was found to be convenient mainly in the analysis of azeliac acid ointment. Thus, commercial formulations containing azelaic acid (ointment, compressed powder, cleansing lotion and lotion) or meglutol (tablets) were subjected to derivatization and HPLC analysis with fluorimetric detection (λ_{exc} = 300 nm; λ_{em} = 460 nm). The results (Table 2) were in agreement with the claimed content for all the drugs. The other ingredients of the formulations did not interfere with the analysis. Benzoic acid present in the ointment did not interfere with azelaic acid analysis, eluting before the drug. The accuracy of the method was verified by analysing commercial samples spiked with known amounts of the drugs (20% of the claimed content); essentially quantitative recoveries (97.5–99.7%) were obtained in each instance.

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