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Single step thin-layer chromatographic method for quantitation of enzymatic formation of fatty acid anilides

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Abstract

The activity of the enzyme involved in catalyzing the formation of fatty acid anilides can be measured by quantitating the fatty acid anilides formed. We have shown earlier that oleic acid is the most preferred substrate among other fatty acids studied for the conjugation with aniline. The reaction product (oleyl anilide) could be separated by thin-layer chromatography (TLC) and then quantified by reversed-phase high-performance liquid chromatography (HPLC). Using [1-^{14}C]oleic acid as substrate, the fatty acid anilide forming activity can be determined in a single step by TLC analysis. The conventional TLC methods used for the separation of the fatty acid esters, however, could not resolve oleyl anilide from the residual [1-^{14}C]oleic acid. Therefore, a simple and reliable TLC method was developed for the separation of oleyl anilide from oleic acid using a freshly prepared solvent consisting of petroleum ether–ethyl acetate–ammonium hydroxide (80:20:1, v/v). Using this solvent system the relative flow (R_f) values were found to be 0.54 for oleyl anilide and 0.34 for aniline, whereas oleic acid remained at the origin. The TLC procedure developed in the present study could be used to determine the fatty acid anilide forming activity using [1-^{14}C]oleic or other fatty acids as substrate and was also found suitable for the analysis of fatty acid anilides from the biological samples. © 1998 Elsevier Science B.V.

Keywords: Fatty acid anilides; Oleyl anilide

1. Introduction

All the case-related cooking oil samples that caused Toxic Oil Syndrome (TOS) were found to contain a significant amount of fatty acid anilides (FAAs), particularly oleyl anilide [1–3]. The levels of oleyl anilide have been found to be associated with the risk of developing TOS [4–6]. Earlier we have shown the formation of FAAs in vitro in the presence of rat liver microsomes [7] and in vivo in rats following the oral administration of aniline [8]. Our studies on enzymic formation of fatty acid anilides using rat liver microsomes showed that oleic acid (18:1) is the most preferred substrate among other fatty acids (16:0, 18:0, 18:2, 18:3 and 20:4) studied [7]. Therefore, fatty acid anilide forming activity can be determined in the tissues and biological specimens using oleic acid as substrate in a two-step procedure; separation by thin-layer chromatography (TLC) on silica gel and quantitation by the reversed-phase high-performance liquid chromatography (HPLC) [9]. The objective of this study was to develop a single-step TLC procedure for separating oleyl anilide from oleic acid and aniline and quantitate oleyl anilide to measure the fatty acid anilide

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forming activity using [1-^{14}C]oleic acid as substrate, as has been used for the fatty acid ethyl ester synthase activity (FAEES) [9].

Earlier, we have reported the separation of individual fatty acid anilides by reversed-phase TLC [10]. This TLC procedure has its limitations, particularly for recovering the reaction product to measure the fatty acid anilide forming activity. Other TLC procedures used for the separation of fatty acid ethyl esters and other lipid conjugates [9,11] could also not resolve oleyl anilide from residual [1-^{14}C]oleic acid. Therefore, a simple and reproducible TLC procedure to separate oleyl anilide from oleic acid is developed and applied for measuring fatty acid anilide forming activity and to separate other fatty acid anilides from the corresponding fatty acids.

2. Experimental

2.1. Chemicals and reagents

Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) and arachidonic (20:4) acids, and aniline were obtained from Sigma (St. Louis, MO, USA). The 16:0, 18:0, 18:1, 18:2, 18:3 and 20:4 fatty acid anilides (FAAs) were synthesized and characterized as described earlier [10]. [1-^{14}C]Oleic acid (53 mCi/mmol) was procured from Dupont NEN (Boston, MA, USA). Frozen rat livers were obtained from PelFreez Biologicals (AR, USA). Silica gel coated glass plates (250 and 500 μm thickness) were purchased from Analtech (Newark, DE, USA). HPLC grade solvents, used for the extraction and TLC separation, were from Fisher Scientific (Fair Lawn, NJ, USA). Unless specified, other biochemicals and reagents were from Sigma.

2.2. TLC separation of oleyl anilide from a mixture of oleic acid and aniline

A mixture of oleyl anilides (50 μg), aniline (10 μg) and oleic acid (50 μg) was subjected to TLC analysis on the 250 μm thick silica gel coated glass plate using the solvent systems [petroleum ether–diethyl ether–acetic acid (75:5:1, v/v) or hexane–diethyl ether–methanol–acetic acid (90:20:5:2, v/v)] used for the separation of fatty acid esters of xenobiotic alcohols [9,11]. Both solvent systems could not resolve the oleyl anilide from oleic acid. Therefore, the petroleum ether–ethyl acetate solvent system was used to separate oleyl anilide from oleic acid and aniline. The most improved separation was observed at a ratio of 4:1 (v/v) of petroleum ether–ethyl acetate as mobile phase, but the anilide fraction still contained traces of residual oleic acid. An addition of 1% ammonium hydroxide to this solvent system improved the separation dramatically; oleic acid remained at the origin and did not migrate at all, also, oleyl anilide was well separated from aniline. A saturated iodine chamber was used to detect the compounds. The purity of the oleyl anilide fraction was further confirmed by the reversed-phase high-performance liquid chromatography (HPLC) using a 334 Beckman liquid chromatograph equipped with 165 variable wavelength UV detector set at 243 nm and a C_{18} column (250×4.6 mm I.D., 5 μm; flow-rate 1.25 ml/min) [10].

2.3. Recovery of oleyl anilide from liver homogenate

Five ml rat liver homogenate [10% (w/v) in 0.1 *M* sodium phosphate buffer (pH 7.2)] with or without oleyl anilide (25 μg) was extracted thrice with three volumes of chloroform–methanol (2:1, v/v). The neutral lipid fraction was separated from phospholipids according to the procedure described previously [12] and subjected to the TLC analysis using the freshly prepared solvent system consisting of petroleum ether–ethyl acetate–ammonium hydroxide (80:20:1, v/v). Silica gel corresponding to the authentic standard of oleyl anilide was scrapped, desorbed with methanol–water (6:1, v/v) and extracted with chloroform. The chloroform layer was dried under nitrogen and redissolved in 100 μl methanol. A known amount of the extract was analyzed by the reversed-phase HPLC as described earlier [10]. The recovery of the oleyl anilide from the liver homogenate was found to be ~80%.

2.4. Enzymatic formation of oleyl anilide

Oleyl anilide forming activity of the rat hepatic microsomes was determined using the TLC procedure developed in the present method. The microsomes were prepared from rat liver according to a previously described method [11] and 0.5, 1 and 2 mg of the microsomal proteins were incubated with 2 μmol of [1-^{14}C]oleic acid (150 dpm/nmol) and 250 μmol aniline in 2 ml of 0.05 *M* sodium phosphate buffer (pH 7.2) at 37°C for 2 h. After incubation, lipids were extracted and subjected to TLC analysis as described above for the liver homogenate. The silica gel corresponding to oleyl anilide was scrapped and extracted with chloroform. A known volume of the chloroform extract was mixed with Tru-Count and the radioactivity was measured using a 1900 CA, Tri-Carb liquid scintillation analyzer. The remaining extract was analyzed by the reversed-phase HPLC method, previously developed for the analysis of FAAs [10]. Fractions of 1.25 ml/min were collected and radioactivity was measured in each fraction as described above.

3. Results and discussion

The fatty acid anilide forming activity, responsible for the conjugation of aniline with endogenous fatty acids, may be a mechanism of retention of aniline and resultant toxicity of aniline and its fatty acid conjugates [6,13–18]. We have shown that the enzyme responsible for the synthesis of FAAs is present in the rat liver microsomes and suggested the role of fatty acid ethyl ester synthase (FAEES) in their biosynthesis [7]. Therefore, the fatty acid anilide forming activity can also be measured similarly as described for FAEES [7,9]. However, the TLC separation of the reaction product (oleyl anilide) from [1-^{14}C]oleic acid by the solvent systems used for the separation of fatty acid ethyl esters showed the presence of [1-^{14}C]oleic acid in the anilide fraction. The relative flow (R_f) values were found to be 0.10 and 0.40 for oleyl anilide, 0.11 and 0.53 for oleic acid and 0.15 and 0.40 for aniline using petroleum ether–diethyl ether–acetic acid (75:5:1, v/v) and petroleum ether–diethyl ether–methanol–acetic acid (90:20:5:2, v/v), respectively (Fig. 1). Although, petroleum ether–ethyl acetate (4:1, v/v), as a solvent system, significantly improved the separation of oleyl anilide from the oleic acid and aniline (Fig. 2), residual [1-^{14}C]oleic acid was still present in the oleyl anilide fraction following a semipreparative TLC on 500 μm thick silica gel coated plates. However, an addition of 1% ammonium hydroxide in petroleum ether–ethyl acetate (4:1, v/v) immobilized the oleic acid at the origin (Fig. 2) and provided very consistent results of [1-^{14}C]oleyl anilide formation following the incubation of a fixed concentration of [1-^{14}C]oleic acid and aniline in the presence of 0.5, 1 and 2 mg/microsomal protein. Radioactivity recovered in the anilide fraction was directly proportional to the concentration of the microsomal protein used for the incubations (Table 1).

Recovery of the TLC separated anilide fraction by reversed-phase HPLC and purity of oleyl anilide from the rat liver homogenate was found to be comparable using the TLC procedure developed in the present study. Separation of a mixture of standard oleyl anilide, oleic acid and aniline, and the neutral lipid fractions obtained from rat liver homogenate with and without standard mixture by semipreparative TLC was found to be very satisfactory (Fig. 3). The reversed-phase HPLC analysis of the anilide fraction obtained from the rat liver homogenate fortified with oleyl anilide showed only one peak corresponding to the retention time of oleyl anilide. No other peaks corresponding to the retention times of other FAAs were observed (data not shown). These results indicate an application of our

Table 1
Enzymatic formation of oleyl anilide in the presence of varying amounts of rat liver microsomal protein

Microsomal protein (mg)	Total DPM corresponding to the anilide fraction by the TLC procedure developed in the present study
0.5	831±254 (5.54)
1.0	1639±400 (10.93)
2.0	2370±177 (15.80)

Values are mean±S.D. of three observations.
Values in parentheses represent equivalent nmol of oleyl anilide formed.

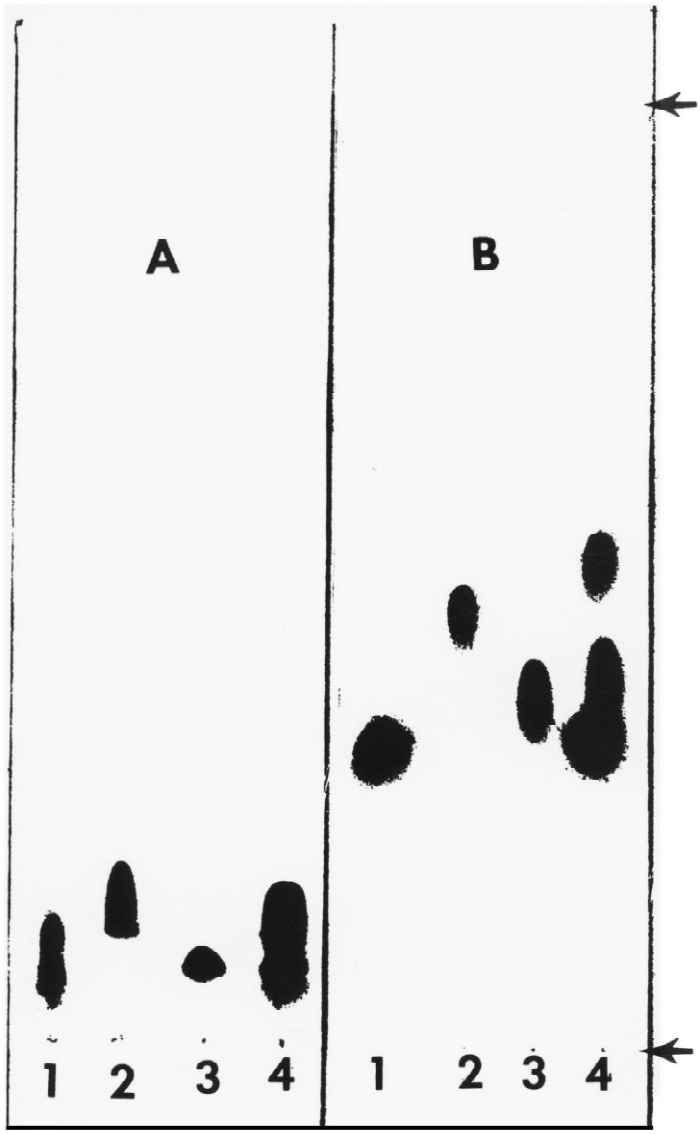

Fig. 1. TLC separation of aniline (1), oleic acid (2) and oleyl anilide (3) and their mixture (4) using the solvent systems: (A) petroleum ether–ethyl ether–acetic acid (75:5:1, v/v) and (B) petroleum ether–ethyl ether–methanol–acetic acid (90:20:5:2, v/v). Note – arrows at top and bottom of the chromatogram indicate solvent front and origin, respectively.

single step TLC procedure, developed in the present study, for the quantitation of FAAs as a measure of fatty acid anilide forming activity. We also found that R_f values for 16:0, 18:0, 18:2, 18:3 and 20:4 fatty acid anilides were the same as that of oleyl anilide, and corresponding fatty acids remained at the origin using the solvent system developed in the present study.

Although, 18:1 (oleyl anilide) was found to be the major product of the incubation of aniline and oleic acid in the presence of rat liver microsomes, a significant amount of 16:0, 18:2, 18:3 and 20:4 fatty acid anilides was also detected (Fig. 4). However, a base line separation of 20:4 fatty acid anilide (peak 2 in Fig. 4A,B) from 18:2 fatty acid anilide could not be achieved under the experimental conditions used

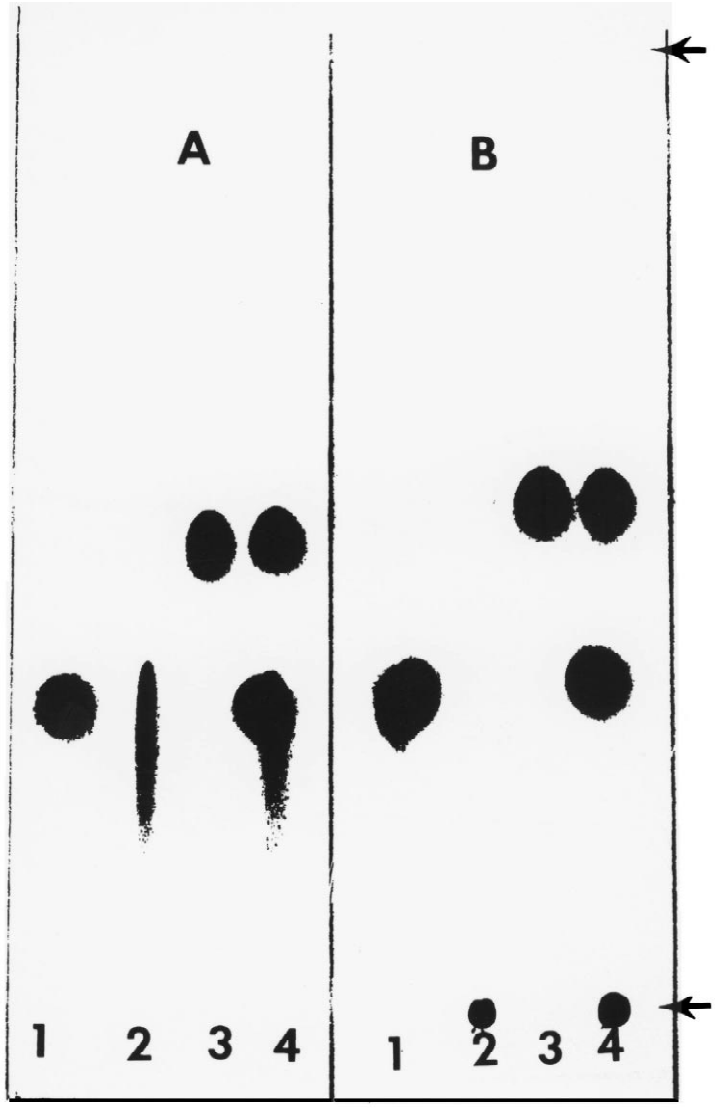

Fig. 2. TLC separation of aniline (1), oleic acid (2) and oleyl anilide (3) and their mixture (4) using the solvent systems: (A) petroleum ether–ethyl acetate (4:1, v/v) and (B) petroleum ether–ethyl acetate–ammonium hydroxide (80:20:1, v/v). Note – see note, Fig. 1.

in the present study. These FAAs may have been formed due to the conjugation of aniline with free fatty acids present in the microsomes. However, another possibility of aniline inducing the hydrolysis of triacylglycerides and/or phospholipids at positions 1 and 2 resulting into availability of more free acids for the conjugation needs to be studied. Approximately 86% of the radioactivity of the anilide fraction recovered from the semipreparative TLC analysis was associated with fractions corresponding to the retention time of oleyl anilide using reversed-phase HPLC (Fig. 4). The radioactivity detected only in the fractions corresponding to the retention of oleyl anilide suggests a reliable recovery of oleyl anilide from residual [1-^{14}C]oleic acid. The presence of a small amount of oleyl anilide in the control microsomal incubation as compared to that detected in the enzymic reaction is also noteworthy. There-

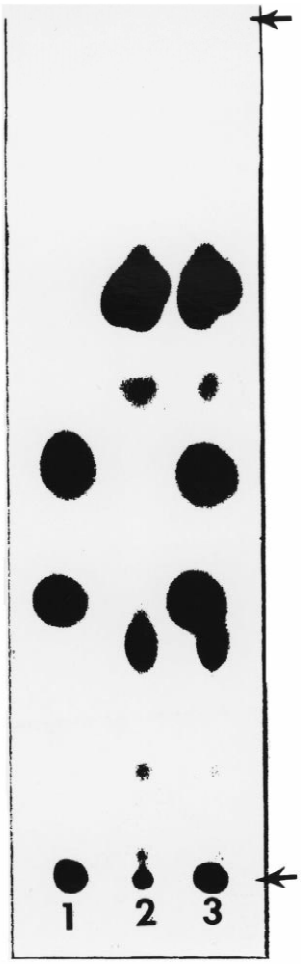

Fig. 3. Semipreparative TLC separation of neutral lipid fraction obtained from rat liver homogenate fortified with a mixture of oleyl anilide, oleic acid and aniline, using petroleum ether–ethyl acetate–ammonium hydroxide (80:20:1, v/v). Lane 1, mixture of standard oleyl anilide (25 μg), aniline (10 μg) and oleic acid (25 μg); lane 2, neutral lipid fraction from control rat liver homogenate; and lane 3, neutral lipid fraction from the rat liver homogenate fortified with a mixture of oleyl anilide, aniline and oleic acid. Note – see note, Fig. 1.

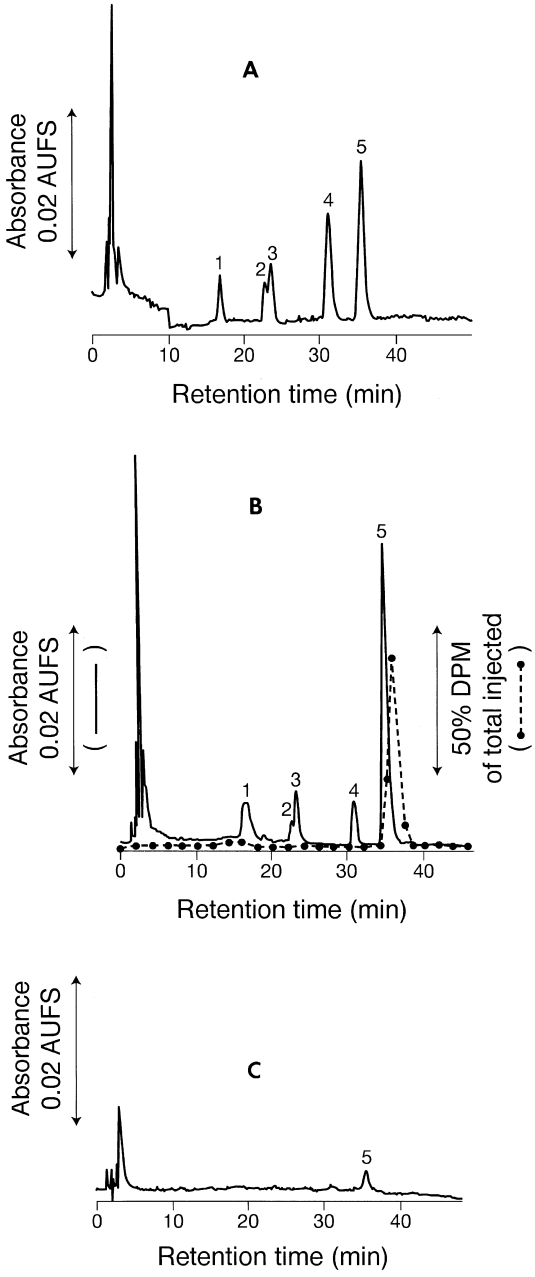

Fig. 4. Reversed-phase HPLC analysis of fatty acid anilide standards (A), and TLC purified fraction corresponding to the oleyl anilide obtained from in vitro incubation of aniline and [1-^{14}C]oleic acid in the presence of rat liver microsomes (B) and control microsomes without aniline (C) (see text for experimental details). Peaks 1, 2, 3, 4 and 5 represent 18:3, 20:4, 18:2, 16:0 and 18:1 fatty acid anilides, respectively. The 20:4 fatty acid anilide is shown as a shoulder (peak 2 in A and B chromatograms) and could not be resolved well from peak 3 of the 18:2 fatty acid anilide.

fore, endogenous formation/presence of the oleyl anilide and other fatty acid anilides also cannot be ruled out. Applicability of this TLC procedure is further demonstrated by measuring the fatty acid anilide forming activity in HepG2 cells and in their quantitation from the tissues (unpublished results).

Acknowledgements

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References

[1] M. Posada, R.M. Philen, I. Abaitua Borda, J.M. Sicilia, A. Gomez, E.M. Kilbourne, Food Chem. Toxicol. 34 (1996) 251.
[2] J.M. Tabuenca, Lancet 2 (1981) 567.
[3] A. Pastana, E. Munoz, Nature 298 (1982) 608.
[4] E.M. Kilbourne, J.T. Bernert Jr., M. Posada, R.H. Hill Jr., I. Abaitua, B.W. Kilbourne, M.M. Jack and the Toxico-Epidemiologic Study Group, Am. J. Epidemiol., 127 (1988) 1210.
[5] M. Posada, R.M. Philen, I. Abaitua, M. Diez, V. Abraira, F. Pozo, R. Pla, M. Pollan, J.M. Sicilia, P. Azpeitia, R.W. Dyer, E.M. Kilbourne, Epidemiology 5 (1994) 404.
[6] M.F. Khan, B.S. Kaphalia, A. Palafox, T.R. Jerrells, G.A.S. Ansari, Arch. Environ. Contam. Toxicol. 21 (1991) 571.
[7] F. Ahmad, B.S. Kaphalia, M.F. Khan, G.A.S. Ansari, Biochem. Arch. 9 (1993) 111.
[8] T.M. Havel, B.S. Kaphalia, M.F. Khan, G.A.S. Ansari, Annual Meeting of Gulf Coast Chapter – Society of Toxicology, University of Health Science Center at San Antonio, TX, 2–4 Nov., 1995.
[9] B.S. Kaphalia, R.R. Fritz, G.A.S. Ansari, Chem. Res. Toxicol. 10 (1997) 211.
[10] B.S. Kaphalia, G.A.S. Ansari, Anal. Toxicol. 15 (1991) 90.
[11] B.S. Kaphalia, G.A.S. Ansari, Mol. Toxicol. 2 (1989) 199.
[12] P. Jauneda, G. Rocquclin, Lipids 20 (1985) 41.
[13] J. Rodrigo, M. Robles, I. Mayo, A. Pastana, A. Marquet, V. Larraga, E. Munoz, Lancet 1 (1983) 414.
[14] P. Sanz, P. Villar, M. Repetto, Vet. Hum. Toxicol. 25 (1983) 401.
[15] R. Pagani, M.T. Portoles, F.G. Gavilanes, P. Garcia-Barreno, A.M. Municio, Biochem. J. 218 (1984) 125.
[16] M.F. Khan, B.S. Kaphalia, A. Palafox, T.R. Jerrells, G.A.S. Ansari, Toxicology 68 (1991) 143.
[17] M.F. Khan, P.J. Boor, B.S. Kaphalia, N.W. Alcock, G.A.S. Ansari, Fund. Appl. Toxicol. 25 (1995) 224.
[18] S.A. Bell, M.V. Hobbs, R.L. Robin, J. Immunol. 148 (1992) 3369.