Artikel Asli/*Original Article*

Determination of Ergosterol as a Potential Biomarker in Pathogenic Medically Important Fungal Isolates
(Penentuan Ergosterol sebagai Biomarker Berpotensi bagi Isolat Fungus Patogenik Berkepentingan Perubatan)

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ABSTRACT

Ergosterol, a component of fungal cell membrane, has been frequently detected as an indicator of fungal presence and mass in environmental samples like soil. However, its detection in major pathogenic fungal species has not been investigated. In this study, the ergosterol contents of ten pathogenic fungal species were determined. Liquid chromatography was used for the detection and quantification of ergosterol extracted from fungal broth cultures. Results showed that ergosterol eluted as a single, well resolved peak in the chromatogram profiles of all tested fungi. Based upon relative amounts of ergosterol produced per fungal mycelial dry weight, three groups of fungal pathogens were identified, namely low ergosterol (Aspergillus niger, Candida albicans and Cryptococcus neoformans at 4.62, 6.29 and 7.08 µg/mg, respectively), medium ergosterol (Fusarium solani, Aspergillus fumigatus, Mucor sp., Penicillium sp., Cryptococcus gattii and Rhizopus sp. at 9.40, 10.79, 10.82, 11.38, 12.60 and 13.40 µg/mg, respectively), and high ergosterol (Candida tropicalis at 22.84 µg/mg), producers. Ergosterol was not detectable in bacterial samples, which were included as controls. This first report on ergosterol detection in major pathogenic fungal species indicates that ergosterol may be used as a biomarker to diagnose invasive fungal infections in clinical samples.

Keywords: HPLC; ergosterol; biomarker; pathogenic; fungus

ABSTRAK

Ergosterol, yang merupakan komponen membran sel fungus, seringkali dikesan sebagai petunjuk kehadiran dan jumlah fungus dalam sampel daripada persekitaran. Walau bagaimanapun, pengesanan ergosterol dalam fungus patogen utama belum dikaji. Dalam kajian ini, kandungan ergosterol dalam sepuluh spesies fungus patogen telah ditentukan. Kaedah kromatografi cecair digunakan untuk mengesan kehadiran dan penentuan kuantiti ergosterol yang diekstrak daripada kultur kaldu fungus. Berdasarkan kuantiti relatif ergosterol yang terhasil pada miselia fungus (berat kering), tiga kumpulan dikenal pasti iaitu mengandungi, ergosterol rendah (Aspergillus niger, Candida albicans dan Cryptococcus neoformans pada 4.62, 6.29 dan 7.08 µg/mg masing-masing), ergosterol sederhana (Fusarium solani, Aspergillus fumigatus, Mucor sp., Penicillium sp., Cryptococcus gattii dan Rhizopus sp. pada 9.40, 10.79, 10.82, 11.38, 12.60 dan 13.40 µg/mg masing-masing) dan ergosterol tinggi (Candida tropicalis pada 22.84 µg/mg). Ergosterol tidak dapat dikesan pada sampel bakteria yang digunakan sebagai kawalan. Laporan pertama pengesanan ergosterol pada fungus patogen utama ini, menunjukkan bahawa ergosterol mungkin boleh digunakan sebagai biomarker untuk pendiagnosan jangkitan fungus invasif dalam sampel klinikal.

Kata kunci: HPLC; ergosterol; biomarker; patogenik; fungus

INTRODUCTION

Ergosterol is a basic sterol forming the cellular membranes of moulds, yeasts and many fungi (Gutarowska et al. 2015). Many correlations between ergosterol content and fungal biomass revealed the specificity of ergosterol as an indicator of fungi to detect and identify fungal colonization and establish their proliferation potential in plants (Chow et al. 2017). Ergosterol quantification is more useful than the direct microscopic count, fluorescence microscopy or staining method, as these methods often lead to under-

or over-estimation of fungal biomass (Chiocchio & Matkovic 2011). The analysis of ergosterol content has been utilized for various applications including assessing agriculture quality (Ruzicka et al. 2000), food deterioration during transportation (Dong et al. 2006), mold growth in buildings and airborne particulates for subsequent predictions of indoor air quality (Hippelein & Rügamer 2004; Axelsson et al. 1995). The first studies on the use of ergosterol as an indicator of the presence of mould in the environment were done by Seitz et al. (1977, 1979). Growth of *Alternaria alternata*, *Aspergillus flavus*, and

Aspergillus amstelodami (*Aspergillus glaucus* group) on moist, milled rice at 25ºC was monitored by ergosterol and chitin assays. Other important grain fungi that were strong ergosterol producers were *Aspergillus candidus, A. ochraceus, Penicillium brevi-compactum, Fusarium moniliforme*, and *F. roseum*. The ergosterol assay therefore was shown to have usefulness for selecting grain hybrids with resistance to weathering and discoloration caused by fungi (Seitz et al. 1977).

The ergosterol assay provides a useful method for quantifying fungal growth, so numerous studies have assessed the relationship between fungal growth and ergosterol production (John & Hocking 2009), however, ergosterol analysis has thus far not been explored for clinical applications as an indicator of fungal infection in patients. Ergosterol detection in plasma and urine of rats has been performed in pharmacokinetic studies of ergosterol in experimental animals (Chen et al. 2013).

The number of opportunistic fungal infections has increased significantly during the past decades, at least in part as the result of a rising number of immunocompromised patients. Individuals at risk for the development of a serious fungal infection include patients undergoing solid-organ, blood and bone marrow transplantation, cancer patients, patients with the acquired immunodeficiency syndrome (AIDS) and other patients receiving immunosuppressive treatment (Pfaller & Diekema 2004; Brakhage 2005). Today, invasive fungal infections are among the most challenging problems in haematology, oncology and intensive care medicine (Vandewoude et al. 2006). Among the approximately 140 000 known fungal species only a few cause human infections (Richardson & Warnock 2003). The most predominant pathogens are the yeast *Candida albicans* and the filamentous fungus *Aspergillus fumigatus*, but other fungal pathogens frequently cause systemic infections, such as the yeast species *C. glabrata*, *C. krusei*, *C. tropicalis*, *Cryptococcus* and *Trichosporon*, filamentous fungi such as *Aspergillus*, *Fusarium*, *Rhizopus* and *Mucor*, and dematiaceous hyphomycetes (Richardson 2005).

Diagnostic assays for invasive fungal infections include detection of biomarkers such as mannan for *Candida* species, galactomannan for *Aspergillus* species (Bašková & Vladimír 2012) and beta-glucan for various fungal species (Karageorgopoulos et al. 2011). Reliable and reproducible diagnostic performance, standardized inter-laboratory procedures, easy application, rapid availability of results and cost-effectiveness are the main features required for the use of a diagnostic test in routine clinical practice. Many of the currently available diagnostic assays for invasive fungal infections lack these features.

Although ergosterol has been detected in fungi that especially contaminate environmental and building samples (Pasanen et al. 1999), its analysis as a biomarker of infection has not been applied to pathogenic fungi. Therefore in this study, the concentration of ergosterol in ten important fungal pathogen species were determined to evaluate its usefulness as a biomarker for fungal infections.

MATERIALS AND METHODS

FUNGI

Ten pathogenic fungal isolates (*Aspergillus fumigatus, Aspergillus niger, Candida albicans, Candida tropicalis, Cryptococcus gattii, Cryptococcus neoformans, Fusarium solani, Mucor* sp.*, Penicillium* sp.*, Rhizopus* sp.) obtained from clinical specimens, maintained on 1.5% (weight/volume, wt/v) potato dextrose agar (PDA) slants, were transferred onto PDA plates and incubated for seven days. At the end of the incubation period, a piece of agar, 5 mm in diameter, was cut aseptically from the active growing edge of each fungus and transferred to 1 L flasks containing 500 mL of potato dextrose broth. The flasks were incubated on a rotary shaker at 125 rpm and 37ºC for 1 week. At the end of the incubation period, the contents of each flask were filtered through sterile gauze. Fungal mycelia trapped in the gauze were washed three times with 100 ml of sterile de-ionized water. The mycelia were freeze-dried. Freeze-dried mycelia were kept in clean sample bottles capped with lined caps and kept in a desiccator until used.

BACTERIA

Four bacterial species (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*) representing Gram positive and Gram negative bacteria frequently encountered in the clinical setting, were subcultured onto nutrient agar for 24 hours and transferred to 500 mL flasks containing 250 mL of nutrient broth. The flasks were incubated on a rotary shaker at 125 rpm and 37ºC for two days. These pathogenic bacterial isolates were included as controls to determine the presence of ergosterol.

EXTRACTION OF ERGOSTEROL

Exactly 100 mg of freeze-dried mycelia and biomass, for each fungal and bacteria species, were blended with 100 mL methanol in a Waring micro-blender and transferred to a 250 mL round bottom flask. The Waring blender was washed with 50 mL methanol and the contents added to the round bottom flask. To the latter, 3.4 g of sodium hydroxide and 50 mL of 95% ethanol were then added and left for one hour at room temperature to saponify the sterol esters. After cooling, the contents were centrifuged at 500 rpm for 30 minutes. The supernatant was transferred to a separatory funnel, 50 mL of water added and extracted twice with 100 mL of pentane. The pentane fraction was concentrated in a rotary evaporator and redissolved in 3 mL of methanol-dichloromethane mixture (1:1). The method of ergosterol

extraction and detection used in this study was according to Dawson-Andoh (2002).

FRACTIONATION AND DETECTION OF ERGOSTEROL

The re-dissolved pentane fraction in methanol-dichloromethane (1:1) was filtered through a 45 µm PTFE membrane filter (CHROMAFIL Xtra) and analyzed by High Performance Chromatography with a Waters 2695 Alliance Separations Modules composed of a quaternary pump, a column oven, and Waters 2487 dual Diode Array Detector (DAD), and Empower software was used for data collection. 20 µL of sample was injected per analysis. The samples were fractionated on an Ascentis C18 HPLC Column (25 cm × 4.6 mm, 5 microns) at a flow rate of 1.5 ml per minute with a methanol-water gradient system as previously published (Dawson-Andoh 2002). The column temperature was maintained at 40ºC and the UV detector was set at 254 nm (0.1 absorbances). The reverse phase column was coupled to a C18 guard column (Waters). Ergosterol (Sigma 45480) external standard at 10, 20, 40, 50, 100, 300, 500, 800 and 1000 µg per mL was used to plot a calibration curve.

RESULTS

Calibration curves of ergosterol standard were found to be linear with the values of 269223, 626850, 1221806, 1312314, 2958753, 10758018, 16089992, 27051242 and 29694650 over the ergosterol standard of 10, 25, 40, 50, 100, 300, 500, 800, and 1000 µg/mL, respectively (Figure 1 & Table 1). The elution time for the ergosterol standard was between 12.991-13.032 minutes. Chromatograms of the ergosterol standard at different concentrations are shown in Figure 2.
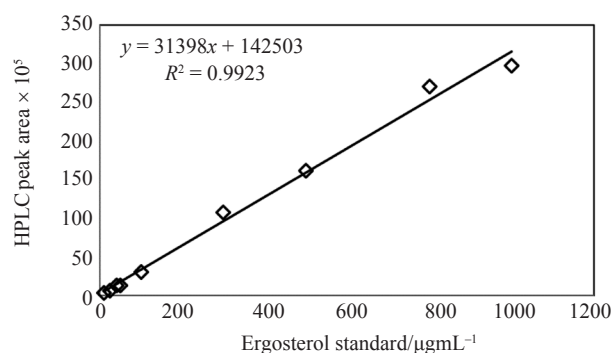

FIGURE 1. Calibration curve of ergosterol standard

TABLE 1. HPLC results for ergosterol standard

Ergosterol Concentration/ µgmL⁻¹	HPLC RT*/ minutes	HPLC peak area	HPLC peak height
1000	13.032	29694650	2285723
800	13.026	27051242	2197821
500	13.026	16089992	1496168
300	13.016	10758018	975950
100	13.001	2958753	258676
50	13.005	1312314	115258
40	12.998	1221806	107711
25	12.994	626850	55205
10	12.991	269223	23596

*RT = retention time

TABLE 2. Ergosterol content of fungal and bacterial isolates determined by HPLC

Sample no.	Isolate	HPLC result	HPLC RT/ minutes	Ergosterol concentration/ µgmL⁻¹	*Ergosterol content in mycelia/µgmg⁻¹
1-Af	*Aspergillus fumigatus*	positive	13.003	359.59	10.79
2-An	*Aspergillus niger*	positive	12.969	153.89	4.62
3-Ca	*Candida albicans*	positive	12.998	209.58	6.29
4-Ct	*Candida tropicalis*	positive	12.975	761.25	22.84
5-Crg	*Cryptococcus gattii*	positive	12.980	419.86	12.60
6-Crn	*Cryptococcus neoformans*	positive	13.007	235.99	7.08
7-Fs	*Fusarium solani*	positive	13.009	313.36	9.40
8-Mu	*Mucor*	positive	13.016	360.72	10.82
9-Pe	*Penicillium*	positive	13.032	379.21	11.38
10-Rh	*Rhizopus*	positive	13.009	446.53	13.40
11-Bs	*Bacillus subtilis*	Negative	-	0	-
12-Ec	*Escherichia coli*	Negative	-	0	-
13-Pse	*Pseudomonas aeruginosa*	Negative	-	0	-
14-Ste	*Staphylococcus aureus*	Negative	-	0	-

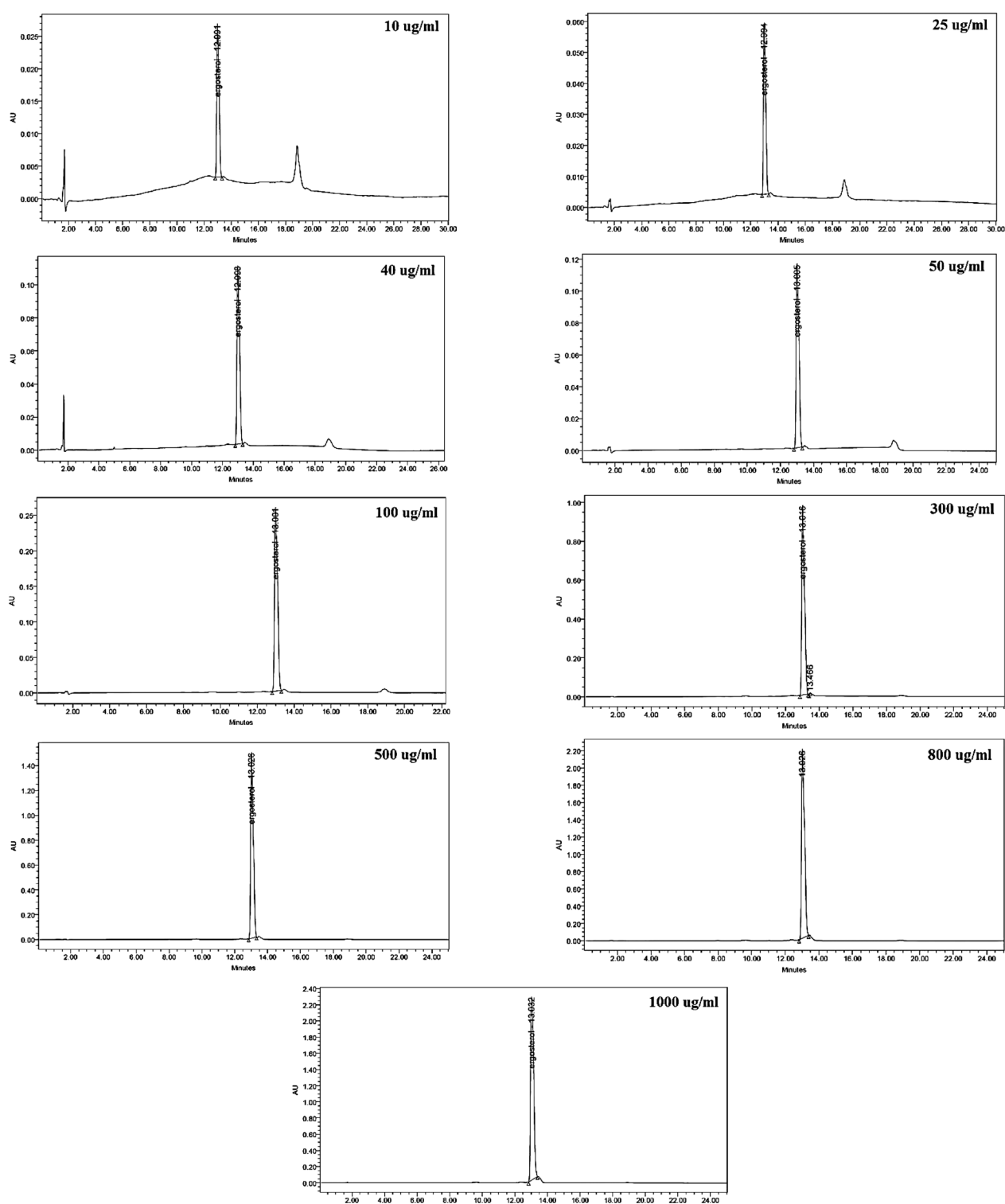*Ergosterol concentration calculated per mg dry mycelial weight

FIGURE 2. Chromatograms of ergosterol standard at varying concentrations

HPLC results of extracts from fungal and bacterial isolates are shown in Table 2. Ergosterol in saponified extracts was identified by comparison of its retention time with that of external ergosterol standard. The ergosterol peak was well resolved and eluted at between 12.969-13.032 minutes. No detectable interfering peaks were observed. *C. tropicalis* and

A. niger yielded the highest and lowest levels of ergosterol respectively. On the basis of relative levels of ergosterol produced, these fungi can be placed into three groups: high ergosterol (*Candida tropicalis*, 22.84 μg/mg), medium ergosterol (*Rhizopus*, *Cryptococcus gattii*, *Penicillium*, *Mucor*, *Aspergillus fumigatus* and *Fusarium solani* at 13.40,

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12.60, 11.38, 10.82, 10.79 and 9.40 µg/mg respectively), and low ergosterol (*Cryptococcus neoformans*, *Candida albicans* and *Aspergillus niger* at 7.08, 6.29 and 4.62 µg/mg respectively) (Table 2). The levels of ergosterol in these fungi were measurable and could therefore be used to detect fungal presence in clinical samples. Bacterial isolates did not show any positive result (no ergosterol peak detected) because they do not contain ergosterol. The bacteria were included in this study as it is necessary to differentiate between bacterial and fungal infections in clinical applications. The chromatograms of ergosterol extracted from pathogenic fungal isolates are shown in Figure 3.
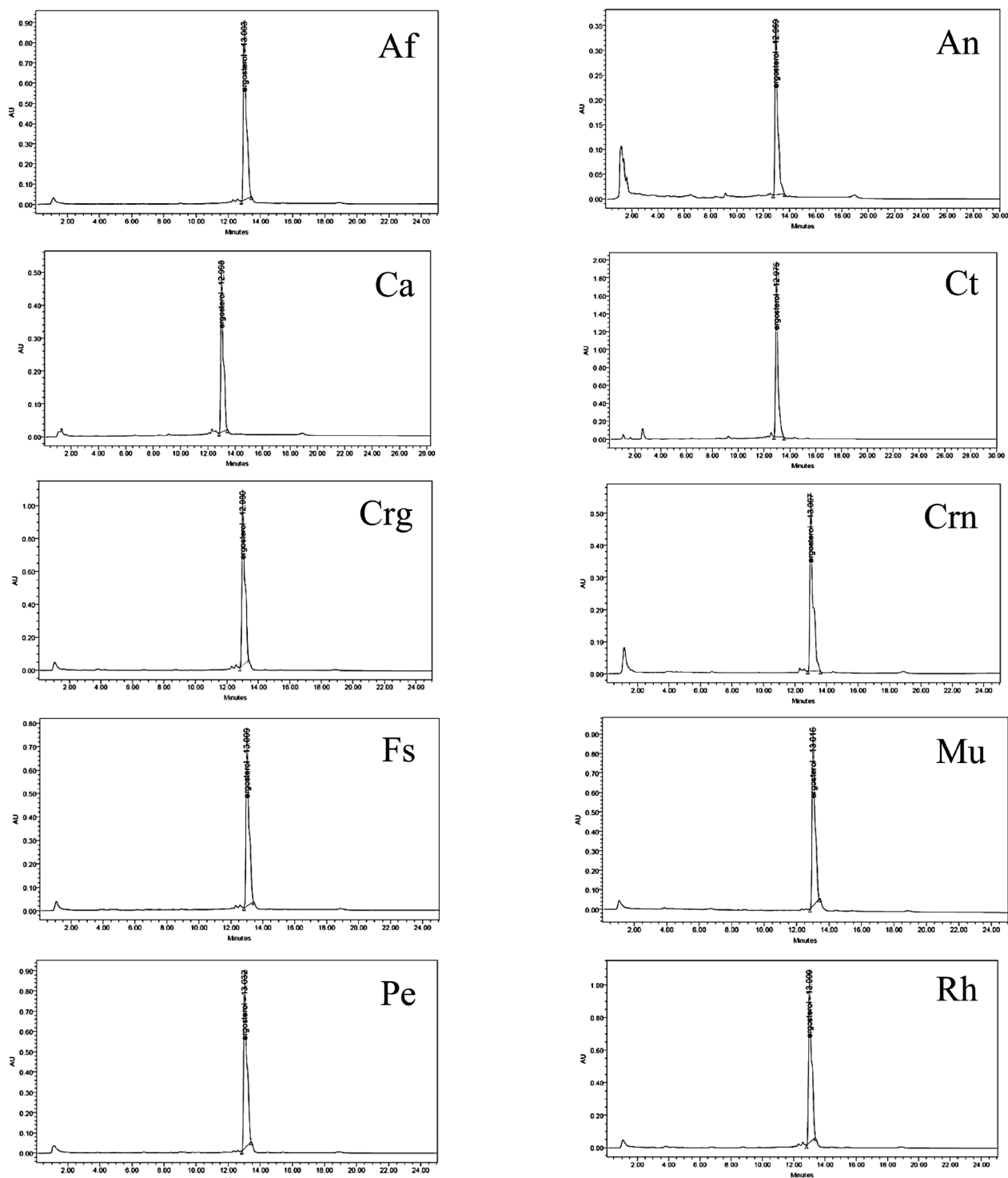

FIGURE 3. Chromatograms of ergosterol in pathogenic fungal isolates studied Af: *Aspergillus fumigatus*; An: *Aspergillus niger*; Ca: *Candida albicans*; Ct: *Candida tropicalis*; Crg: *Cryptococcus gattii*; Crn: *Cryptococcus neoformans*; Fs: *Fusarium solani*; Mu: *Mucor* sp.; Pe: *Penicillium* sp.; Rh: *Rhizopus* sp.

DISCUSSION

Although ergosterol is specific to fungi and may be used as a relative index of associated fungal biomass, its concentration does not always correlate with absolute fungal biomass (Gessner & Chauvet 1993). Concentration of ergosterol in a fungus is influenced by both internal and external factors, such as fungal species (Huang et al. 1985; Newell et al. 1987; Salmanowicz & Nylund 1988), physiological state of fungus, its age, developmental stage (Huang et al. 1985), general growth conditions, chemical composition of substrate, temperature and pH of the environment (Weete 1980; Arnezeder & Hampel 1991; Olsen 1973; Vanden 1990). However, in contrast to large differences in ergosterol content of macrofungal fruiting bodies reported by Huang et al. (1985), Gessner & Chauvet (1993) observed very little dependence of ergosterol content of fourteen strains of aquatic hyphomycetes commonly found on decaying leaves on external factors such as media composition and age of culture. Although ergosterol content may vary in a fungal species under differing conditions, for clinical applications, it is more relevant that ergosterol is detectable in a patient as this may have both diagnostic and prognostic value.

In the present study, we have shown that the ergosterol content in pathogenic fungi is measurable and varies in different species. Ergosterol is a structural component of the fungal cell membrane and there have been no reports linking its content to fungal pathogenicity. We have also shown that ergosterol is not detectable in bacterial isolates, therefore it may be a highly specific indicator of fungal presence. Determination of ergosterol content may indicate the susceptibility of the fungus to antifungal drugs, as enzymes involved in the synthesis of ergosterol are the target of azole antifungal drugs. A previous study had shown that the quantitation of ergosterol content in *C. albicans* isolates, following exposure to the azole drug fluconazole, was useful for discerning susceptibility to the drug, enabling the discrimination between resistant and highly resistant isolates (Arthington-Skaggs et al. 1999) and appeared more predictive of in vivo outcome compared to conventional susceptibility testing in an animal study (Arthington-Skaggs et al. 2000). In resistant *C. albicans* isolates, ergosterol content did not decrease as much as in sensitive isolates, following fluconazole treatment (Arthington-Skaggs et al. 1999). Based on these results, therefore detection and quantification of ergosterol periodically in a patient with invasive fungal infection may be useful for therapeutic drug monitoring, especially when azole antifungal drugs are prescribed.

CONCLUSION

In conclusion, the detection of ergosterol in clinical specimens may be a very useful biomarker especially for the diagnosis of invasive fungal infections.

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